In vitro Fermentation of β (1→3) Glucans Using Human Fecal Bacteria: An Evaluation of Their Prebiotic Potential

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Abstract

Prebiotics are non-digestible food components (mainly non-starch polysaccharides and oligosaccharides) that beneficially affect the host by stimulating the growth and/or activity of one or a limited number of bacteria, such as *Bifidobacteria* and other lactic acid bacteria in the colon. In the present study, the prebiotic potential, in terms of *in vitro* fermentability and bifidogenic effect, of some commercially available native and chemically modified (by carboxymethylation) β-glucans, as well as those prepared from mushroom sclerotium were studied.

Commercial β-glucans with different molecular weight were investigated. They included native pachyman (PAC), curdlan (CUR), laminarian (LAM), carboxymethlyated pachyman (CM-PAC) and curdlan (CM-CUR). Prebiotic potential of mushroom β-glucan extracted by sonication from *Poria cocos* (PC) sclerotium (PSS), which consisted of more than 85% non-digestible carbohydrates (NDC), was also studied. Mushroom oligosaccharides prepared from PSS (PCO) by enzymatic digestion using glucanases were also investigated to find out if reduction in the degree of polymerization (DP) of the β-glucans could enhance their prebiotic potential. The chemical structure of PSS, PAC and CUR were confirmed to be pure linear β (1→3) glucans, while LAM consisted of a β-glucan having 1→6 branches on its 1→3 backbone, with small amounts of other monosaccharides (< 10%).

The *in vitro* fermentability of β-glucans was evaluated by the organic matter disappearance (OMD) as well as the amount of short chain fatty acids (SCFAs) produced as analyzed by gas chromatography (GC), during a 24h *in vitro* fermenation using human fecal microflora as inoculum. The changes in the total

count of bacteria and the number of *Bifidobacteria* during the *in vitro* fermentation were enumerated by fluorescent *in situ* hybridization (FISH). Fructo-oligosaccharides (FOS) and cellulose were acted as the positive and negative control, respectively.

All β-glucans tested were found to possess different degree of prebiotic potential. The OMD ranged from 22.75 ± 5.82% in PSS to 66.59 ± 5.64% in LAM, in which the OMD of LAM, PCO, CM-CUR and CUR were found to be significantly higher than the negative control cellulose (p <0.05). Total SCFA production ranged from -0.48 ± 0.16 mmol per gram of original organic matter (OM) in PSS to 5.80 ± 1.09 mmol per gram of original OM in LAM. The OMD and total SCFA production of LAM and PCO after 24h *in vitro* fermentation were found to be significantly higher than that of PAC and CUR ($p < 0.05$). Carboxymethylation of PAC and CUR enhanced the *in vitro* fermentability, as observed in CM-PAC and CM-CUR, but the effect was not statistically significant ($p > 0.05$). β-glucans extracted from PC sclerotium (PSS and PAC) showed the lowest OMD and SCFA production, but the *in vitro* fermentability of PSS was significantly increased when it was enzymatically hydrolyzed to oligosaccharides (DP 2 to 10), as observed in PCO ($p < 0.05$). The molar ratio of acetate, propionate and butyrate within 24h *in vitro* fermentation of different β-glucans varied, with an average molar ratio of 23: 33: 15.

The treatment of LAM and PCO led to an approximately three-fold increase of *Bifidobacteria* and were found to be significantly higher than other β-glucans ($p <$ 0.05). Carboxymethylation enhanced the bifidogenic effect of PAC and CUR, as observed in CM-PAC and CM-CUR, but the effect was not significant ($p > 0.05$). Reducing DP of PSS, on the other hand, led to a significant increase in the

bifidogenic effect of this β-glucan (PCO) ($p < 0.05$).

To conclude, β (1→3) glucans possess prebiotic potential that vary according to their chemical structures (especially molecular weight). β (1→3) glucans with low DP generally have higher prebiotic potential than those with high DP. Enzymatic digestion (partial hydrolysis by glucanase) might serve as an effective method in preparing novel potent prebiotics with optimal DP from natural sources of β glucans.

摘要

化學益生素 (也稱前生素，益生原) 是一種非消化性的食物成份 (主要爲非澱粉多糖或寡糖)，到達大腸後可選擇性地被大腸內的有益菌 (如雙歧桿菌和其他乳酸菌) 利用，從而刺激有益菌的生長或活動，產生對宿主有益的影響。在這研究中，我們所選擇的葡聚糖 (β-glucan)，包括市面上一些天然和經過化學加工，以及由食用菌的菌核所提取的，進行體外可發酵性及雙歧效果這兩方面的實驗，評估這些β葡聚糖作爲化學益生素的能力。

被研究的β葡聚糖包括市面上未經加工的茯苓聚糖 (PAC)、凝膠多糖 (CUR) 和海帶多糖 (LAM)，及經化學加工 (酸甲基化) 的茯苓聚糖 (CM-PAC) 和凝膠多糖 (CM-CUR)，均屬於具有不同分子量的β (1→3)葡聚糖。天然茯苓菌核含有非常豐富的非消化性碳水化合物 (>85%)，因此本研究亦會探討由天然茯苓菌核提取的β葡聚糖 (PSS)的化學益生素能力，。此外，爲了解調低聚合度 (DP) 對 β葡聚糖的化學益生素能力的影響，本研究會利用葡聚糖酶分解 PSS，並提取當中的茯苓寡糖 (PCO)進行研究。PSS、PAC 及 CUR 均被確認爲無分枝的β (1→3)葡聚糖，而 LAM 則在β (1→3)骨幹上有少量 β (1→6)分枝，亦含有少量其他的糖 (<10%)。

本研究利用人類糞便細菌進行上述β葡聚糖的二十四小時體外發酵實驗，並量度當中的有機物質消耗 (OMD) 及以氣相色譜法量度短鏈脂肪酸 (SCFA)的產生，從而評估這些β葡聚的體外可發酵性。此外，亦會用螢光原位雜合 (FISH) 測量於二十四小時體外發酵實驗中的細菌總量及雙歧桿菌數量的改變。在實驗中，低聚果糖 (FOS) 及纖維素會分別用作陽性及陰性對照。

上述的β (1→3)葡聚糖都被發現有不同程度的化學益生素能力。當中 PSS 的

有機物質消耗最低，爲 22.75 ± 5.82%，而最高則爲 LAM 的 66.59 ± 5.64%。LAM ，PCO ，CM-CUR 及 CUR 的有機物質消耗都顯著高於纖維素 ($p < 0.05$)。在短鏈脂肪酸生產量方面，最低的爲 PSS 的每克原有機物質 -0.48 ± 0.16mmol，而最高則爲 LAM 的每克原有機物質 5.80 ± 1.09 mmol。LAM 和 PCO 在二十四小時體外發酵實驗的有機物質消耗和總短鏈脂肪酸生產量顯著較 PAC 和 CUR 高 ($p < 0.05$)。酸甲基化能加強 PAC 和 CUR 的體外可發酵性，但其效果並不明顯 ($p > 0.05$)。 雖然由茯苓菌核提取的β葡聚糖 (PSS 和 PAC) 的體外可發酵性是眾多β葡聚糖中最低的，但當 PSS 被葡聚糖酶降解爲 DP 2 至 10 的(PCO) 寡糖後，其體外可發酵性卻被顯著提升 ($p < 0.05$)。另外，在β葡聚糖的二十四小時體外發酵實驗中，乙酸、丙酸及丁酸的摩爾比值各有不同，但普遍來說也是維持在 23：33：15。

在 LAM 和 PCO 的二十四小時體外發酵實驗後，雙歧桿菌的數量上升近三倍，相對於其他β葡聚糖，亦有顯著上升 ($p < 0.05$)。酸甲基化能加強 PAC 和 CUR 的雙歧效果，但升幅並不顯著 ($p > 0.05$)。PCO 的雙歧果效亦明顯較 PSS 的爲高 ($p < 0.05$)。

總的來說，β (1→3)葡聚糖擁有化學益生素能力，而該能力則受不同因素，特別是分子量影響。通常 DP 較低的β (1→3)葡聚糖都會比 DP 較高的β (1→3)葡聚糖有較高的化學益生素能力。本研究亦證實利用葡聚糖酶將天然高 DP 的 β 葡聚糖降解爲低 DP β葡聚糖，是發展新種類化學益生素的其中一個有效方法。

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Abbreviations

Bovine serum albumin (BSA)

Carboxymethylated pachyman (CM-PAC)

Carboxymethylated curdlan (CM-CUR)

Curdlan (CUR)

Degree of polymerization (DP)

Denaturing gel electrophoresis (DGGE)

4',6-diamidino-2-phenylindole (DAPI)

Dietary fiber (DF)

Dimethyl sulfoxide (DMSO)

Fluorescent *in situ* hybridization (FISH)

Fructo-oligosaccharides (FOS)

Gas chromatography (GC)

Gas chromatography-mass spectrometry (GC-MS)

Gastrointestinal tract (GIT)

Laminarian (LAM)

Non-digestable carbohydrates (NDCs)

Non-digestable oligosaccharides (NDOs)

Non-digestable polysaccharides (NDPs)

Pachyman (PAC)

Organic matter (OM)

Organic matter disappearance (OMD)

Partially methylated alditol acetates (PMAAs)

Polymerase chain reaction (PCR)

Poria cocos (PC)

Poria cocos sclerotium oligosaccharide (PCO)

Relative viscosity (η_{rel})

Resistant starch (RS)

Short chain fatty acids (SCFAs)

Sonication fraction of *Poria cocos* sclerotium (PSS)

Specific viscosity (η_{sp})

Total dietary fiber (TDF)

Trichloroacetic acid (TCA)

Content

Chapter 1 Introduction

1.1 Colonic fermentation

1.1.1 The large intestine and the intestinal microflora

In Western populations, human large intestine in general lengths approximately one meter in adults, with an internal surface area in the region of about 1300cm^2 (Banwell *et al.*, 1981). The large intestine typically receives about 1.5kg materials from the small intestine every day. Most of these materials are water that are rapidly absorbed; the remains consists of various dietary components that have escaped digestion in the small intestine, together with a range of host-derived materials (Degnan, 1993).

The human large intestine is actually colonized by a complex microbial community that is often referred to as the intestinal microflora. There could be up to 10^{14} microbial cells reside within the colonic epithelium, and is 10 to 20 times the total number of tissue cells in the entire body (Suau *et al.*, 1999). Different types of indigested materials entering the colon from the small intestine, therefore, serve as substrates for microbial fermentation.

The actual number of bacterial species that colonize the human large intestine is controversial. In 1995, Gibson and Roberfroid suggested that there were at least 400 culturable bacterial species in the human large intestine (Gibson and Roberfroid,

1995). More recently, it has been estimated that there are more than five hundred species coexist in the human colon (Hughes *et al.*, 2001), although statistical extrapolations from the 16S rDNA sequencing of cloned amplicons derived from human fecal community DNA from one patient suggested that there was less than 150 operational taxonomic units (defined as differing in 16S rDNA sequence by less than 2%) (Suau *et al.*, 1999). Within this large community, however, some 40 species make up ~99% of the population (Mai & Morris, 2004). The predominant species, as revealed by quantitative fluorescence *in situ* hybridization (FISH) using 16S rRNA targeted probes, are found to be predominantly the *Bacterioides-Prevotella* group (a Gram-negative anaerobe), the *Clostridium coccoides* group and the *Clostridium leptum* group (Gram-postive anaerobes) (Franks *et al.*, 1998; Sighir *et al.*, 2000), in which all of these groups were recently found to be the predominant ones (Franks *et al.*, 1998).

Human intestinal microflora with its metabolic activity e.g. fermentation, is possibly associated with many health-related functions such as maintenance of gut homeostasis, metabolism of xenobiotics and stimulation of gut immunity (Macfarlane & Macfarlane, 2003).

1.1.2 Major substrates and products of colonic fermentation

When we are discussing about the human large intestine, "fermentation" is loosely used to describe the great variety of reactions and the overall metabolic processes involved in the anaerobic breakdown of organic matter.

Basically, substrates for colonic microflora fermentation are those dietary ingredients that resist enzymatic digestion in the small intestine. Normally, in the small intestine, fats are digested by 90 – 95% while the digestion of proteins is less complete which is estimated that about 3 – 9g pass into the colon daily (Macfarlane & Cummings, 1991). On the other hand, various types of carbohydrates are digested to different degrees. For instance, monosaccharides are absorbed efficiently (>90%) when consumed in physiological amounts (Priebe *et al.*, 2002). Conversely, non-digestable carbohydrates (NDC) like dietary fiber (DF), resistant starch (RS), other non-digestible polysaccharides (NDP) and non-digestible oligosaccharides (NDO), form a large proportion as substrates for colonic fermentation. The human large intestine is thus a multi-substrate environment that supports a highly diverse community of microorganisms.

The two main types of fermentation that are carried out in the gut are proteolytic and saccharolytic. Generally, saccharolytic fermentation is more favorable than a proteolytic fermentation in terms of the types of metabolites produced. The end-products of proteolytic fermentation include nitrogenous metabolites (such as phenolic compounds, amines and ammonia), some of which are carcinogenic (Smith & Macfarlane, 1996). Large intestinal fermentation using carbohydrates as substrates, on the other hand, would generate different types of products, including hydrogen, carbon dioxide, methane, other gases and short chain fatty acids (SCFAs), the major anions in colonic contents (Priebe *et al.*, 2002). These reactions are carried out by intestinal microflora which possess glycosidases capable of hydrolyzing the glycosidic linkages of oligo- and polysaccharides not digested or incompletely digested by the enzymes in the upper gastrointestinal tract (GIT). These intestinal microflora are thus able to metabolize the resulting

monosaccharides. An increased production of these SCFAs leads to a decreased luminal pH (Priebe *et al.*, 2002). SCFAs are the principal products of anaerobic microbial fermentation in the large intestine and affect colonic health by providing energy to the epithelial cells. In pure culture, different anaerobic gut bacteria can produce a wide variety of products, including acetate, propionate, butyrate, succinate, lactate and ethanol, but only the first three are normally found as major products in the mixed gut ecosystem. This is largely explained by the fact that products such as lactate and succinate are efficiently utilized by certain groups of anaerobic bacteria (Macfarlane & Macfarlane, 2003). (See Fig. 1.1)

The production of the SCFAs including acetate, proprionate and butyrate is considered as being beneficial to the host. The vast majority of SCFAs (>95%) formed are quickly absorbed and metabolized by the host. This allows the salvage of energy from undigested food in the upper GIT, and can account for up to 9% of the host energy requirements (Hume, 1995). Both acetate and propionate are absorbed from the colonic lumen. Propionate is transported to the liver for gluconeogenesis and acetate to various tissues as a fuel. Butyrate is oxidized by the colonic epithelium (Priebe *et al.*, 2002). SCFAs have a wide range of physiological functions in the body, including colonocyte metabolism (Roediger, 1980), cell growth and differentiation (deFazio *et al.*, 1992), epithelial cell transport (del Castillo *et al.*, 1994), metabolism of lipids and carbohydrates in the liver (Demigne & Remesy, 1994), intestinal motility (Cherbut *et al.*, 1996), energy generation in muscle, kidney, heart and brain (Macfarlane & Cummings, 1991).

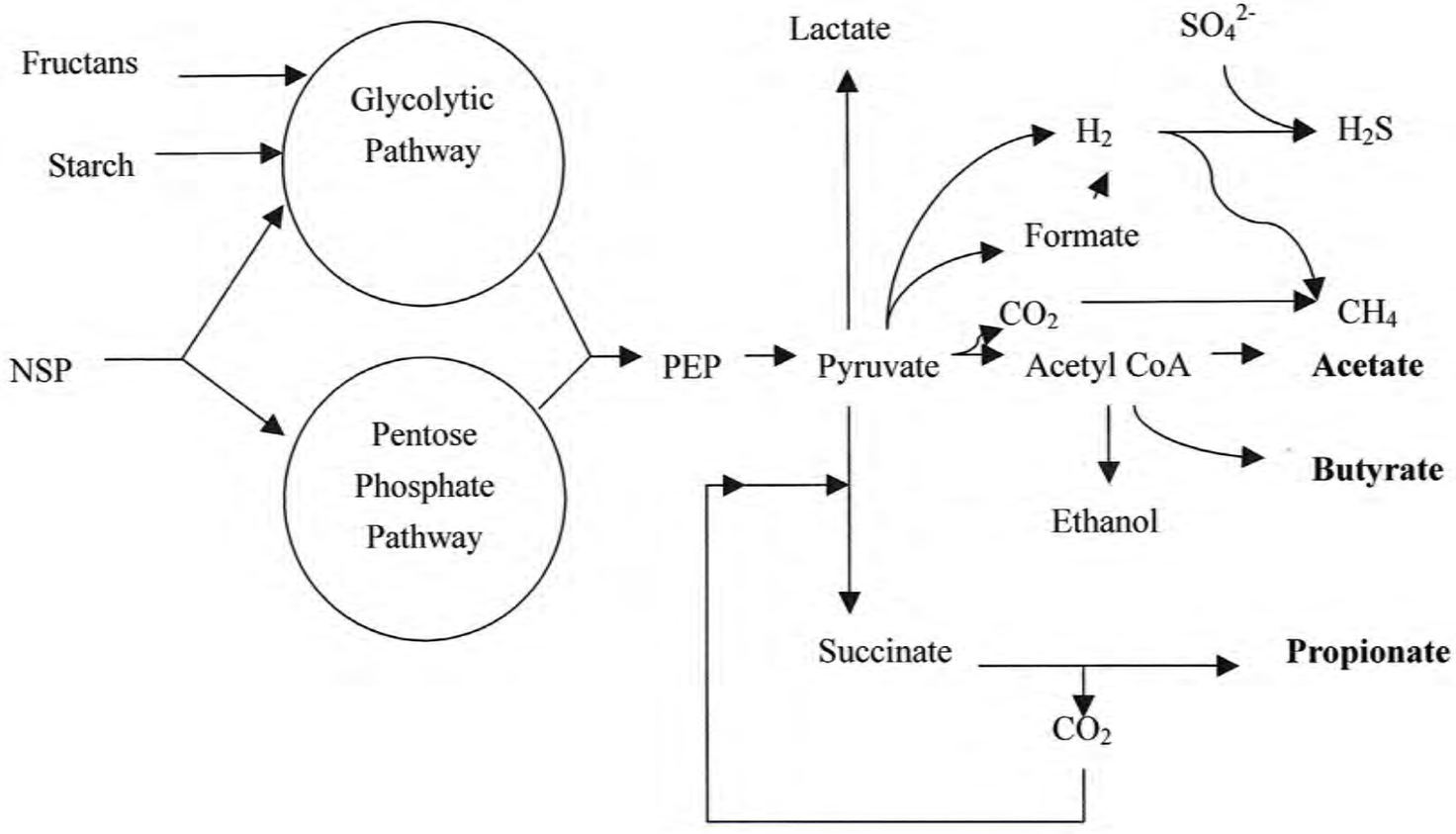

Fig 1.1 Metabolic fates of starch and different NSPs in the human large intestine. (Adapted from Macfarlane & Macfarlane (2003)).

1.1.3 Beneficial bacteria

One of the major targets of probiotics or prebiotics (see 1.2.1) is definitely *Bifidobacteria.* Lactic acid-producing genera like the *Bifidobacteria* or *Lactobacilli* have long standing "health image". Within these, the most significant microorganisms are believed to be the *Bifidobacteria. Bifidobacteria spp.* are normal inhabitants of the human and animal gut, and newborns are colonized within days after birth. The population seems to be relatively stable until advanced age when it has been reported to decline. Although the population of *Bifidobacteria* in the intestine is relatively stable, it would be influenced by diet, antibiotics, stress etc (Gibson *et al.*, 2003)

Bifidobacteria are the major component of the microbial barrier to infection (Gibson & Roberfroid, 1995). They produce a range of antimicrobial agents that are active against Gram-positive and -negative microbes. *Lactobacilli* are also healthy bacteria and produce a range of antimicrobial agents, but they are present in relatively smaller amount in human large intestine. In addition to producing antimicrobial agents, a large proportion of beneficial bacteria competitively excludes pathogens by occupying receptor sites and competing for space and nutrients (Gibson & Wang, 1994b). *Bifidobacteria* have also been reported to have beneficial effects on specific immune functions. Bacterial fermenting activity in the human colon has been claimed to have a beneficial impact on maintaining health. Predominant substrates for these bacterial fermentations include not only non-starch polysaccharides, resistant starch and oligosaccharides, but also undigested proteins, sugar alcohols and endogenous compounds such as pancreatic secretions and mucins.

1.2 Prebiotics

1.2.1 Definitions of probiotics and prebiotics

Probiotics are live microorganisms that exert health beneficial effects upon ingestion in certain numbers, and have been used to modify the intestinal microflora for a long time. More recently, prebiotics, which are defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson & Roberfroid, 1995), have been used in the functional food industry.

Therefore, for substrates to be classified as prebiotics, the following three criteria should be met, (1) the substrate must not be hydrolyzed or absorbed in the small intestine; (2) it must be selective for beneficial commensal bacteria in the colon by encouraging the growth/metabolism of them; and (3) it will alter the microflora to a healthy composition by inducing beneficial luminal/systemic effects within the host (Gibson & Fuller, 2000).

From this point of view, the human large intestine contains indigenous bacteria, some are beneficial and health-promoting, and others may be detrimental to the host's health. Prebiotics should enter the colon intactly, undergo specific metabolism therein and direct toward beneficial bacteria rather than harmful species. This would ultimately leads to a marked compositional change within these bacterial populations. Preferred target microorganisms for prebiotics basically include species that belong to *Bifidobacteria* and *Lactobacillus* genera. Efficient prebiotics may also lead to the

suppression of pathogenic species such as *Clostridia* (Gibson & Roberfroid, 1995).

1.2.2 General characteristics of prebiotics

Although numerous materials enter the large intestine (e.g. unabsorbed sugars and sugar alcohols, oligosaccharides, chitin and amino-sugars), the major substrates for microbial fermentation are the carbohydrates that resist the small intestinal digestion (details refer to 1.1.2). Thus, they are always the major targets for prebiotic investigations.

Carbohydrates can loosely be classified as digestible or non-digestible carbohydrates (NDC). Digestible carbohydrates are the various sugar-containing molecules that can be digested by amylase or the saccharidases of the small intestine to sugars that can be absorbed from the intestine (Macfarlane & Macfarlane, 2003). The predominant digestible carbohydrates in foods include starch, sucrose, lactose (in milk) and maltose. NDC, on the other hand, cannot be digested by the enzymes in the small intestine and are the primary component of dietary fiber. Most of these non-starch polysaccharides are part of the plant cell wall and they possess beta-glycosidic linkages instead of the alpha ones as in the case of starch. These beta linkages would not be hydrolyzed by the small intestinal enzymes like amylases which can only hydrolyze alpha linkages. Therefore, NDP and NDO serve as the primary substrate for growth of the microbes in the large intestine and contribute to stool formation and laxation, and are thus potential prebiotic candidates.

1.2.3 Current studies on prebiotics

1.2.3.1 Non-β-glucan typed prebiotics

Any food substrate that enters the large intestine is a potential prebiotic; however, its selective fermentation by the intestinal microflora is the most crucial factor. They are currently conceived of all carbohydrates with relatively short-chain length, especially oligosaccharides with the degree of polymerization from 2 to 10. These NDO seem to be preferred prebiotics. Gibson and Fuller (2000) have summarized the NDO for which *in vitro* and *in vivo* data have been published to support their prebiotic effect. These include lactulose, inulin-type fructans, galacto-oligosaccharides, soy-bean oligosaccharides (raffinose and stachyose), iso-maltooligosaccharides and xylooligosaccharides (Table 1.1). Most of these are not β-glucans.

For example, the effects of the prolonged administration of transgalacto-oligosaccharides for 21 days on *Bifidobacteria* and fermentative activity of colonic flora were assessed by Bouhnik *et al.* (1997). All the 8 volunteers showed a significant increase in fecal concentrations of *Bifidobacteria* from 8.6 ± 0.6 to 9.7 ± 0.5, 9.7 ± 0.6 and 9.5 ± 0.6 $\log_{10}$ cfu/g on day 1, 7, 14 and 21, respectively ($P < 0.05$). Hopkins and co-workers (1998) tested the prebiotic potential of 15 carbohydrates. They showed that galactooligosaccharides and oligofructose, with a low degree of polymerization, supported best growth of the test micro-organisms (including *Bifidobacteria spp.*). In contrast, xylooligosaccharides and pyrodextrins were almost invariably poor bifidobacterial substrates (Hopkins *et al.*, 1998).

Table 1.1 Examples of *in vitro* and *in vivo* studies designed to determine the efficacy of prebiotic candidates

Potential prebiotics	*In vitro* study	*In vivo* study
Lactulose	Fadden & Owen (1992)	
Fructo-oligosaccharides	Wang & Gibson (1993)	Williams *et al.* (1994)
	Gibson & Wang (1994a & b)	Bouhnik *et al.* (1994)
	McBain & Macfarlane (1997)	Gibson *et al.* (1995)
		Buddington *et al.* (1996)
		Kleesen *et al.* (1997)
Galacto-oligosaccharides	Durand *et al.* (1992)	Ito *et al.* (1990)
	Bouhnik *et al.* (1997)	Rowland & Tanaka (1993)
		Bouhnik *et al.* (1997)
Soybean oligosaccharides	Hayakawa *et al.* (1990)	Hayakawa *et al.* (1990)
	Saito *et al.* (1992)	
Iso-maltooligosaccharides		Kaneko *et al.* (1994)
Xylo-oligosaccharides	Hopkins *et al.* (1998)	Okazaki *et al* (1990)

Source of information: Gibson & Fuller, 2000

There are a number of other different studies on the prebiotic effect of non-β-glucans. Bacterial counts for *Bifidobacteria* increased by a mean of 2.8 ± 0.57 $\log_{10}$ cfu/g feces after 3 weeks of supplementation comprising 8g of fructo-oligosaccharides (FOS) per day, but soon declined afterwards without the supplementation of FOS (Guigoz *et al.*, 2002). In volunteers treated with lactulose for 30 days, *Bifidobacterium spp.* increased significantly (p <0.01) over the pre-treatment samples, from 8.8 ± 0.5 $\log_{10}$ cells/g feces to 9.3 ± 0.3 $\log_{10}$ cells/g feces. The increase was also found to be significantly different (p <0.01) from the

Bifidobacteria number in the placebo group over the same period (Tuohy *et al.*, 2002). Dextran and oligodextrans, which composed of α-glycodic bonds, were also found to display a bifidogenic effect in batch fermentation cultures, with high levels of persistence up to 48h (Olano-Martin *et al.*, 2000). Moreover, galacto-oligosaccharides was found to lead to an increase of 0.85 $\log_{10}$ cfu/ ml batch culture and 0.54 $\log_{10}$ cfu/ ml batch culture in *Bifidobacteria* and *Lactobacillus* number, respectively, upon 24h *in vitro* fermentation using human fecal inoculum (Tzortzis *et al.*, 2004).

1.2.3.2 β-glucan type prebiotics

Currently recognized prebiotics are NDC with a wide range of monosaccharide components and linkages. The most widely used prebiotics include fructo-oligosaccharides and inulin, which are heterogeneous carbohydrates with their structures α-D-Glc-(1→2)-[β-D-Fru-(1→2)-]$_n$ (Playne & Crittenden, 1996). Glucose is the major monosaccharide constituent of most of the other NDC having prebiotic effects. Examples are malto-oligosaccharides, [α-D-Glc-(1→4)]$_n$ where n = 2 – 7, isomalto-oligosaccharides, [α-D-Glc-(1→6)]$_n$ where n = 2 – 5, cyclodextrins, [-α-D-Glc-(1→4)-]$_n$ cyclical where n = 6 – 12. Nonetheless, researches on the prebiotic potential of glucans with β glycosidic linkages are relatively rare. One of these is gentio-oligosaccharides [β-D-Glc-(1→6)]$_n$ where n = 2 -5 (Playne & Crittenden, 1996). There are currently lack of scientific researches focused on the prebiotic effect of other β-glucans, especially those with 1→3 glycosidic linkages.

1.3 Potential β-glucan type prebiotics

1.3.1 Commercial sources of β (1→3) glucans as potential prebiotics

Beta-glucans are β (1→3)-linked polymers of glucose that are produced as fungal cell wall constituents and are also released into the extracellular milieu (Mueller *et al.*, 2000).β (1→3) glucans have been reported to possess varies physiological effects that are beneficial to the host. β (1→3) glucan has been proven to trigger non-specific immune response by both stimulating and activating macrophage cells (Rasmussen *et al.*, 1992). It has also been reported that β (1→3) glucans may possess antitumor activities (Mimura *et al.*, 1985). It is Generally Recognized As Safe (category GRAS according to FDA) and has no toxicity or side effects. Therefore, β (1→3) glucans serve as one of the potential targets for other physiological effects, such as *in vitro* fermentability and prebiotic effect.

In this project, several commercially available β (1→3) glucans of different sources and variable DP would be subjected to *in vitro* fermentability and prebiotic investigations. On the other hand, polysaccharides and oligosaccharides isolated from the sclerotia of a medicinal mushroom, *Poria cocos*, would also be studied, so as to get an evaluation of the potential of β (1→3) glucans to act as prebiotics.

1.3.1.1 Pachyman (PAC) and carboxymethylated-pachyman (CM-PAC)

Pachyman is a β (1→3) glucan derived from *Poria cocos* sclerotia (Basidiomycetes). It is reported to contain approximately 100% 1,3-linked D-glucosyl residues. It is an off-white powder and is water insoluble. In order to improve the water solubility of PAC, commercial manufacturers (Megazyme) synthesized (CM-PAC) by carboxymethylating highly purified pachyman with chloroacetic acid. It was a tan colored powder with a degree of carboxymethylation of approximately 0.2. As given by the manufacturer, CM-PAC was found to form a colloidal suspension in water or buffer at 0.5% w/v.

1.3.1.2 Curdlan (CUR) and carboxymethylated curdlan (CM-CUR)

Curdlan, produced by *Alcaligenes faecalis* var. myxogenes, is a linear polysaccharide composed of glucose units with β (1→3) bond (Harada *et al.*, 1968). Curdlan is basically insoluble in water and would gel on heating. It is extensively used as an additive for texture improvement in food industry. Studies about the prebiotic potential, mainly bifidogenic property, of curdlan have been established in murine models (Shimizu *et al.*, 2001), but few investigations have been carried out using human fecal microflora. Curdlan was produced by *Alcaligenes faecalis* var. myxogenes 10C3K. It is reported to contain approximately 100% 1,3-linked D-glucosyl residues (Megazyme). It is a white powder and is water insoluble. In order to improve the water solubility of curdlan for more diverse application, commercial suppliers (Megazyme) have prepared carboxymethylated curdlan (CM-CUR) by carboxymethylation of highly purified curdlan with chloroacetic acid. It is a light tan

colored powder with a degree of carboxymethylation of approximately 0.4. CM-curdlan was found to form a highly viscous aqueous solution in water or buffer at 0.5% w/v.

1.3.1.3 Laminarian (LAM)

Laminarian are polysaccharides extracted from certain algae like *Laminaria digita*. Laminarian is also called laminarin or laminaran, which is a well characterized β-D-(1→3) glucans with β-D-(1→6) side chain branching on average every tenth glucose subunit along the polymer backbone (Williams *et al.*, 1991).

1.3.2 Non-digestible carbohydrates (NDC) from mushroom

1.3.2.1 Mushroom sclerotia as a good source of β-glucan

According to Chang and Miles (2004), "a mushroom is a macrofungus with a distinctive fruiting body which can be either epigeous (above ground) or hypogeous (under ground) and large enough to be seen with the naked eye and to be picked by hand." Mushrooms belong to only two subdivisions of fungi; the vast majority are basidiomycetes, and a few are ascomycetes.

Generally speaking, the mushroom life cycle could be divided into four main stages, spore, mycelium, primodium and sclerotium as well as the fruiting body. Sclerotia are a resting phase in the mushroom life cycle having a hardened tuber and wood-like in texture. While in this dormant state, the mushroom species can survive

unflavorable weather conditions like drought and fire. When the conditions become favorable for growth, the sclerotia would uptake water and become softened. Fruiting bodies would then emerge directly from the sclerotia, starting a new life cycle (Chang & Miles, 2004).

In the view of being a potential prebiotic, sclerotia are definitely the most suitable stage in the life cycle of edible mushrooms. Owing to its high fiber content when compared to mycelium and fruiting body, sclerotia provide a good source of NDP (especially β-glucans) for microbial fermentation. While, fruiting bodies of edible mushroom generally consist of only 3% to 32% NDP (Breene, 1990). The NDP content of the sclerotial stage of certain edible mushrooms, including PC, could reach to above 80% of the total dry matter (Wong *et al.*, 2003).

1.3.2.2 *Poria cocos* (PC) sclerotia

PC is one of the commonly used edible mushrooms with therapeutic effects in Chinese Medicine. It belongs to the class Basidiomycota. The Basidiomycotina are also called "club fungi" as their spores are attached to club-shaped structures named *basidia* (Hobbs, 1995). Many well-known medicinal genera are Basidiomycotina, including *Boletus*, *Agaricus*, *Amanita*, and *Polyporus*. Different scientific researches have shown that certain types of Basidiomycotina possess antitumor, antibiotic, antiviral, antiallergic, immunostimulating, hypoglycemic, hypocholesterolemic and blood pressure-lowing properties (Ooi, 2000).

PC is one of the earliest recorded and the most commonly used fungus of all

Chinese medicinal herbs (Liu & Bau, 1980). It is also known as "hoelen" in Chinese, while "Fuling" is the Chinese name for its sclerotium. The sclerotium of PC is spherical, oval or irregular in size with a diameter of 10 – 30 cm. Sclerotium of PC can be collected all year round, especially in August and September (Liu & Bau, 1980). According to traditional Chinese medicine, PC is considered as mild sweet and bland. It is used to cure edema and clear febrile illness. The cortex of PC sclerotium is used as a diuretic and a decoction for cough, whereas the internal white portion is used to relieve uneasiness arising from pregnancy and the heart discomfort. The sliced or whole sclerotium is often applied to treat jaundice and to induce menstruation. The polysaccharides of PC such as pachyman and pachymaran exhibit strong antitumor and immunodulatory activities (Chang & Miles, 2004).

Upon common extraction methods of PC sclerotium, including the use of saline, hot water and alkaline, fractions of different yields and proportions of proteins, polysaccharides and minerals would be resulted. With the use of relatively dilute alkaline solution as the extraction solvent and sonication to increase the yield, the extraction of PC sclerotium would yield linear β (1→3) glucan. As the main fraction of PC sclerotium, its yield could reach approximately 84% of its original dry matter (Wang *et al.*, 2004). Therefore, PC sclerotium serve as a good source of NDC while its sonicated fraction in alkaline medium provide a good source of β (1→3) glucan.

1.3.2.3 Oligosaccharide preparation from PC sclerotium

An effect of molecular size was observed in different *in vitro* and *in vivo* studies, with the oligosaccharides having a higher fermentability or prebiotic effect

than the polysaccharides they derived from. Pectic-oligosaccharides were found to be a better prebiotic candidate than the pectins in a pH controlled batch mixed fecal cultures (Olano-Martin *et al.*, 2002). In a murine model, *Bifidobacteria* showed higher ability to utilize fructans of low DP (FOS with n= 2 to 4 and oligofructans with n = 2 to 8) than those with higher DP (inulin with n >23) (Bielecka *et al.*, 2002).

There are numerous methods that are used for oligosaccharide preparation. These include enzymatic hydrolysis, chemical hydrolysis as well as enzymatic synthesis. Within these three methods, enzymatic hydrolysis and synthesis are more commonly used, as the process is usually better controlled. FOS is produced using these two approaches. Enzymatic synthesis is carried out using the disaccharide sucrose as the starting material. With the transfructosylation activity of the enzyme β-fructofuranosidase (EC 3.2.1.26), FOS formed contain between 2 and 4 β (1→2) linked fructosyl units linked to a terminal α-D-glucose residue (Ghazi *et al*, 2005). Another method used is the controlled enzymatic hydrolysis of the polysaccharide inulin using inulinase (Playne & Crittenden, 1996). The products closely resemble those formed from sucrose-transfructosylation, but the chain length would usually be longer (with n = 2 to 8) (Jin *et al.*, 2004). Galacto-oligosaccharides were mostly produced by enzymatic synthesis rather than hydrolysis. Patent activity in process development for the manufacture of galacto-oligosaccharides from lactose continue to be high (Playne & Crittenden, 1996). Del-Val and Otero (2003) made use of a biphasic aqueous media containing polyethylene glycol for the enzymatic synthesis of oligosaccharides from lactose.

Chemical hydrolysis is a less commonly used method for oligosaccharide

preparation when compared to the enzymatic approaches, as it is found to be more difficult to control. Hydrolysis of oat β-glucans using chemical and enzymatic method were compared (Johansson *et al.*, 2005), in which no degradation was observed at all or complete breakdown to glucose occurred. In this study, hydrolysis with lichenase was found to be more easily controlled.

Therefore, controlled enzymatic approach was selected for more specific action on the hydrolysis of the glycosidic linkages of PC sclerotium. Enzyme would be selected based on the structural analysis of the original as well as the sonicated fraction of PC sclerotium.

1.4 Microbial analysis by molecular methods

In vitro fermentation using human fecal inoculums is the most commonly used method for testing potential prebiotics. In order to determine whether the treatment of the potential compounds would lead to a change in fecal microbial composition, different methods have been developed for the identification and quantification of target microbes (Tannock, 2003).

1.4.1 Traditional cultural techniques

One major prerequisite for studying the effect of a certain compound on the intestinal microflora is an accurate identification and quantification of them. There are several methods that based on different principles including plate counts, molecular techniques, fluorescence and immunological techniques. Until the late 90s, the first method was the most widely used. Investigations of gut microflora at that time involved the use of culture techniques using selective bacteriological culture media, microscopy and the determination of the fermentative and other biochemical capabilities of bacterial isolates (Hartemink & Rombouts, 1999). Tables 1.2 and 1.3 show the commonly used media for culturing and enumerating the total anaerobes and *Bifidobacteria.*

Table 1.2 Media used for the detection of total anaerobes from feces

Medium	Reference
BB-Agar	(Summanen *et al.*, 1993)
Brain Heart Infusion Blood Agar	(Roberts *et a*l., 1992)
Columbia Blood Agar	(Bartram *et al.*, 1994)
Eggert Gagnon Agar	(Mitsuoka *et al.*, 1973)
RCA	(Venketeshwer Rao *et al.*, 1994)
Wensinck Agar	(Wensinck *et al.*, 1981)
Wilkins Chalgren Agar	(Bearne *et al.*, 1990)

Source of information: Hartemink & Rombouts, 1999

Table 1.3 Media used for the detection of *Bifidobacteria* from feces

Medium	Reference
Bifidobacterium selective agar (BS-Agar)	(Mitsuoka *et al.*, 1973)
Bifidobacterium selective medium (BBM-agar)	(Cole & Fuller., 1989)
Bifidus Blood Agar	(Rasic, 1984)
Bifidobacterium Iodoacetate Medium (BIM-25 Agar)	(Munoa & Pares, 1988)
China Blue Agar	(Mevissen-Verhage *et al.*, 1987)
Liver Cystine Lactose Agar (LCL-agar)	(Rasic, 1984)
Modified Rogosa Agar	(Rasic, 1984)
MPN-agar	(Tanaka & Mutai, 1980)
MRS	(Venketeshwer Rao *et al.*, 1994)
MRS Agar with LiCl and antibiotics (MRS-NN)	(Norin *et al.*, 1991)
Neomycin Paromomycin Lithium Naldixic acid Agar (NPNL)	(Teraguchi *et al.*, 1978)
Propionate agar (Beerens-Agar)	(Beerens, 1990)
Raffinose-Bifidobacterium Agar	(Hartemink *et al.*, 1996)
Reinforced Clostridial Agar with Cephalothin and Blood (RCB)	(Yaeshima *et al.*, 1997)
Rogosa agar	(Rasic, 1984)
Tomato Casein Peptone Yeast Agar (TCPY)	(Rasic, 1984)
Tomato Casein Peptone Yeast Agar (TCPY) with azide	(Rasic, 1984)
Tomato Casein Peptone Yeast Agar (TCPY) with sorbic acid	(Rasic, 1984)
Tomato Casein Peptone Yeast Agar (TCPY) with antibiotics	(Roberts *et al.*, 1992)
Transgalactosyloligosaccharide Agar (TOS-agar)	(Sonoike *et al.*, 1986)
x-Gal medium	(Chevalier *et al.*, 1991)
YN-6 Agar	(Resnick & Levin, 1981)

Source of information: Hartemink & Rombouts, 1999

These selective bacteriological media have been essential in studying the diversity of colonic bacteria as they allow isolation, identification (by further identification techniques) and most importantly, quantification of bacteria to be made. Nevertheless, since the predominant microbes that inhabit the human large intestine are obligately anaerobic, these culture-dependent techniques require special apparatus, equipment and facilities which help maintaining a strictly anaerobic environment for the cultivation of the extremely oxygen-sensitive species colonized within the gut (Tannock, 2003). Besides being laborious, time-consuming and costly (Blaut *et al.*, 2002), there are also two important problems using this technique. Firstly, nowadays, as more and more phylogenetic information of these interested colonic microbes have been established, we know that few culture media used in the analysis of the microflora are absolutely selective and not all of the species within a population could grow equally well on such selective media, leading to biased results. More importantly, it was recently revealed that a large proportion of intestinal and fecal bacteria are non-culturable (Langendijk *et al.*, 1995; Suau *et al.*, 1999). Although most information about the bacterial community in the human GIT has been obtained by selective cultivation of microbes from fecal samples, recent culture-independent approaches in which the sequence variability of the 16S rRNA genes has been used illustrated that most of the predominant bacteria in human fecal samples have not yet been obtained in culture (Suau *et al.*, 1999). Table 1.4 shows the cell count of fecal sample using different identification methods.

Table 1.4 Total microscopic count of human fecal sample by (1) DAPI staining, (2) FISH using bac 338 probe, (3) culturing on anaerobic non-selective medium

	(Total cell count) Cells/g		
	Dry weight	Wet weight	% of total cell count
(1)DAPI staining	$10.6 \times 10^{11} \pm 0.4 \times 10^{11}$	$2.72 \times 10^{11} \pm 0.10 \times 10^{11}$	
(2) Bac 338	$7.1 \times 10^{11} \pm 0.2 \times 10^{11}$	--	67%
(3) Brain heart infusion medium	$2.2 \times 10^{11} \pm 0.2 \times 10^{11}$	$0.56 \times 10^{11} \pm 0.05 \times 10^{11}$	21%

Source of information: Suau *et al.*, 1999

Table 1.4 showed the comparison of total cell count of human fecal sample made by three methods, (1) 4', 6-diamidino-2-phenylindole (DAPI) (a non-selective nucleic acid stain) staining; (2) hybridization using the Bact338 oligonucleotide probe, which basically binds to all bacteria and thus represented the number of bacteria detected by this molecular method; and (3) anaerobic non-selective brain heart infusion medium to grow cultural fecal bacteria. When compared to the total cell count, culturable species only accounted for about 21%, while that by molecular methods was about 67% (Suau *et al.*, 1999). Although some of these DAPI-stained cells might be non-viable, it was likely that many were viable but non-culturable due to their fastidious requirements for anaerobiosis, or more likely, due to the complex nutritional interactions that could occur between the inhabitants of bacterial communities. These nutritional complexities may be difficult, if not impossible, to be achieved in laboratory culture media.

1.4.2 Newly emerging molecular techniques

Advances in the field of molecular phylogeny have made it possible to study bacterial populations by a culture-independent approach. The first widely used

molecular technique in microbial systematics, which still required culturing of the respective bacteria, was total genomic DNA hybridization. Instead of using small genomic regions, this approach utilizes the whole genomes to determine the degree of similarity between two microbes. Total genomic DNA hybridization formed the basis for molecular microbial phylogeny before the advent of the 16S rDNA revolution. Molecular tools based on 16S rDNA sequence similarities such as FISH, denaturing gel electrophoresis (DGGE), quantitative dot blot hybridization, restriction fragment length polymorphism and large scale 16S rDNA sequencing have helped to overcome limitations of conventional microbiological plating methods in studying the fecal microflora composition (Tannock, 1999; Vaughan *et al.*, 2000).

Although the use of 16S rDNA-directed techniques, cloning and sequencing have provided new insights into the bacterial composition of the human GI tract, these approaches are all based on PCR amplification methods and hence the results cannot be accurately converted to the actual number of bacteria. Molecular techniques, especially FISH performed with 16S rRNA-targeted oligonucleotide probes has been shown to be a very powerful tool for both detecting and enumerating interested microbes in environmental and fecal samples (Amann *et al.*, 1995).

It has been revealed that small ribosomal subunit RNA (16S rRNA in the case of bacteria) contained regions of highly conserved nucleotide base sequence and are interspersed with hypervariable regions (V regions). These hypervariable regions contained the signatures of phylogenetic groups and even species. Members of the colonic microflora can thus be accurately identified by extraction of DNA from a

pure bacterial culture, polymerase chain reaction (PCR) amplification of the 16S rRNA gene using universal primers that target conserved bacterial sequences and determination of its nucleotide base sequences. The 16S rRNA has been the most widely employed molecule to develop the phylogeny of prokaryotes. Within the various regions of 16S rRNA, the degree of conservation differs considerably. Analyses of rRNA sequences have revealed signature sequences, short stretches of rRNA, that are unique to a certain group or groups of organisms enabling the phylogenetic placement and identification of bacteria (Woese, 1987).

Whole cell hybridization combined with specific 16S rRNA-targeted oligonucleotide probes detects morphologically intact cells. Since fluorescently labeled probes are often applied, this technique is often referred to as fluorescence *in situ* hybridization (FISH) (Suau *et al.*, 1999). In principal, bacterial cells are permeabilised by treatment with paraformaldehyde or ethanol to allow the probes to reach their target, the ribosomal RNA. If the 16S rRNA contains a sequence complementary to the probe sequence and if this target sequence is accessible to the fluorescent oligonucleotide, a hybrid is formed causing the whole cell to fluoresce. Since each cell contains several thousand ribosomes, the fluorescing cells can be visualized by epifluorescence microscopy. The major advantage of this method is the capability to detect individual cells in a complex mixture of cells without the need to grow them.

Over the years, FISH has been modified and adapted to identify and enumerate fecal microflora. More and more phylogenetic sequences have also been worked out for bacterial identification. Phylogenetic analysis of rRNA genes, amplified by

polymerization chain reaction (PCR), has been used as a major and efficient method to investigate the bio-diversity of intestinal microflora and revealed many novel species (Suau *et al.*, 1999; Zoetendal *et al.*, 1998). Langendijk and his colleagues (1995) first developed rRNA-targeted probes for *Bifidobacteria*. It was followed by the design of rRNA-targeted probes targeting *Clostridium leptum* subgroup (Sghir *et al.*, 2000) and *Eubacterium spp.* (Schwiertz *et al.*, 2000) in human feces. For better understanding of the composition of fecal microflora, Harmsen and his co-workers (2002) also tried to develop a set of probes for detecting *Phascolarctobacterium* and relatives (Phasco741), *Veillonella* (Veil223), *Eubacterium hallii* and relatives (Ehal469), *Lachnospira* and relatives (Lach571) and *Eubacterium cylindroids* and relatives (Ecyl387), which were usually not the most concerned ones.

With the more extensive development on the techniques and probe diversity, FISH therefore serves as a good tool for fecal composition study. FISH is also desirable and suitable for investigating the effect of a prebiotic candidate on fecal microflora before and after fermentation.

1.5 Objectives and significance of the present study

The present study aims at evaluating the potential of β (1→3) glucans as novel prebiotics. The objectives of the project are as follows,

1. To prepare glucose-based β (1→ 3) oligosaccharides of degree of polymerization (DP) 2 to 10 from the sonicated fraction of PC sclerotium (PSS) by enzymatic hydrolysis;
2. To investigate the prebiotic effect of PSS and oligosaccharides prepared from PSS by studying their *in vitro* fermentability and bifidogenic properties;
3. To investigate the prebiotic effect of commercial β-(1→3) polysaccharides with different molecular weights and from different sources (pachyman [PAC], curdlan [CUR] and laminarian [LAM]) as well as the effect of carboxymethylation to PAC and CUR by comparing their *in vitro* fermentability and bifidogenic effect.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Commercial β-glucans

Besides laminarian (LAM) (Sigma), all other polysaccharides, including pachyman (PAC), carboxymethylated-pachyman (CM-PAC), curdlan (CUR) and carboxymethylated-curdlan (CM-CUR) were purchased from Megazyme International Ireland Ltd., in powder form.

2.1.2 β-glucan from *Poria cocos* sclerotium

Poria cocos sclerotium (Fig. 2.1) was purchased from a local pharmacy and was claimed to be originated from the Yunnan Province in Mainland China. After removal of the brown sclerotial peel, the white body was milled to powder by a mechanical grinder (MF 10, IKA® Works, Japan) using a 0.5mm sieve.

Twenty grams of the *Poria cocos* sclerotium powder was refluxed with 300 ml ethanol (80%) for an hour to remove simple sugars and other low molecular weight substances. After centrifugation of 10000 rpm for 30 min, the pellet was soaked in NaOH (1M) in a ratio 1: 50 for 48 h at ambient temperature. After that, the mixture was subjected to sonication (VCX 600, Sonics) with amplitude 40% for 40 min in an ice bath and was then centrifuged at 10000 rpm for 30 min. The supernatant was dialyzed to allow salt removal and was finally lyophilized to obtain the dry sonication fraction of *Poria cocos* sclerotium (PSS).

Fig 2.1 *Poria cocos* (PC) sclerotia

2.2 Chemical characterization of *Poria cocos* sclerotium

2.2.1 Lowry method for soluble protein determination

The basic procedure of this soluble protein determination method was modified according to Lowry *et al.* (1951), which was then manufactured as a kit by Sigma, Co., and was used in this experiment.

2.2.1.1 Reagents

The Lowry procedure was used for determination of soluble protein in *Poria cocos* sclerotium as well as PSS using Protein Assay Kit (Sigma, Procedure no. P5656). The Sigma Protein Assay Kit consists of Lowry Reagent, Modified. (Sigma L 1013), DOC Solution (Sigma D 5525), which is an aqueous solution of sodium deoxycholate (1.5mg/ml), Trichloroacetic Acid Solution (TCA) (Sigma T4396), which is an aqueous solution of trichloroacetic acid (72% w/v), FOLIN & CIOCALTEU's phenol reagent (Sigma F9252) and Protein Standard (Sigma P 7656), which is a bovine serum albumin, fraction V (BSA).

2.2.1.2 Determination of soluble protein content

Standard solutions of bovine serum albumin (BSA) (Sigma) (25 – 200μg protein/ ml) were prepared. About 15mg *Poria cocos* sclerotium or PSS were dissolved in 10 ml distilled water and used as the sample stock solution. BSA standard or sample solutions (1 ml) were well mixed with 1 ml of Lowry Reagent and the mixture was stood at room temperature for 20 min. FOLIN &

CIOCALTEU's phenol reagent (0.5ml) was then added to each tube with rapid and immediate vortex-mixing. The mixture was allowed to stand at room temperature for 30 min for color development. Following the same procedure mentioned above, a reagent blank was prepared with distilled water used instead of the sample solution. The protein content in the solution was determined by measuring its absorbance at 750 nm with reference to a calibration curve constructed from BSA.

2.2.2 Total sugar content analysis (Phenol-sulphuric acid method)

The method for total sugar content determination was modified according to Dubois *et al.* (1956). In order to test for the total sugar content, acid hydrolysates of water-insoluble samples were prepared by depolymerisation. About 15 mg of sample was hydrolyzed with 0.7 ml 12M sulphuric acid at 35℃ for 1h with stirring. The hydrolysate was diluted to 2M with 3.5ml of distilled water and heated in a boiling water-bath with shaking for 1 h. The hydrolysate was cooled to room temperature. The hydrolysate (10μl) was diluted 50-fold using 2M sulphuric acid. This was then vortex-mixed with 0.5ml of 5% phenol in water. After rapid addition of 2.5ml concentrated sulphuric acid and votex-mixed, the mixture was allowed to stand for 30 min at room temperature. Using 2M sulphuric acid as the blank, the amount of sugar was measured by reading absorbance at 490nm. A calibration curve was made using glucose standard (Supelco) (0 – 100 μg/ml).

2.2.3 Total dietary fiber analysis

The total dietary fiber (TDF) content of the original *Poria cocos* sclerotium and

PSS were assessed by the Official Methods of Analysis (991.43) (AOAC, 1995) with some modifications.

2.2.3.1 Digestible carbohydrate and protein removal by enzyme treatment

About one gram of the original *Poria cocos* sclerotium powder or PSS were added to 50 ml MES/Tris buffer (0.05M, pH 8.2) and well mixed. Heat-stable α-amylase (0.1 ml) (Sigma A-3306) was added and the mixture was incubated in a 95 ℃ to 100 ℃ water-bath for 15 min. The solution was then cooled to room temperature. Protease (50mg/ml, 0.1ml) (Sigma P-3910) was then added and the solution was incubated at 60℃ for 30 min with continuous shaking. After cooling to room temperature, the pH of the solution was adjusted to the range of 4.1 to 4.8, with the use of 0.325M HCl. This was followed by the addition of amyloglucosidase (0.3 ml) (Sigma A-9913) from *Aspergillus niger*, and the mixture was incubated at 60℃ for another 30 min with continuous shaking.

2.2.3.2 Total dietary fiber content determination

The enzyme digestate was then mixed with four volumes of 95% ethanol and kept at room temperature overnight for the precipitation of soluble dietary fiber. The ethanol-digestate mixture was then vacuum-filtered (Fibertec System E 1023 Filtration Module, Tecator) and the residue was collected by a pre-weighed crucible. The residue was washed in a sequence of 78% ethanol (3 times, 20 ml each), 95% ethanol (2 times, 10 ml each) and lastly acetone (2 times, 10 ml each). The residue, together with the crucible was dried in a 105 ℃ oven overnight and weighed.

Following the same procedure mentioned above, a reagent blank was prepared without any sample added. The weight of TDF was corrected for its residual protein (g) and ash (g) as well as the reagent blank (g). Total dietary fiber content was calculated according to the following fomula and the TDF content was expressed as percentage of sample dry weight,

$$\text{TDF (\%)} = \frac{(\text{weight}_{\text{TDF}} - \text{weight}_{\text{residual protein}} - \text{weight}_{\text{residual ash}} - \text{weight}_{\text{reagent blank}}) \times 100\%}{\text{Dry weight}_{\text{sample}}}$$

For residual protein determination, the Official Methods of Analysis (955.04) (AOAC, 1995) with some modifications was used. One of the sample residues was digested with 12.5 ml concentrated sulfuric acid at 420℃ for about two hours. The residue was allowed to cool and was then diluted with 75 ml distilled water. The solution was then distilled with 10M sodium hydroxide solution and ammonia released was collected with 2% boric acid solution. This was then titrated with standardized 0.1M hydrochloric acid. The total nitrogen content of the sample was then calculated using the formula shown as below. The residual protein content was obtained by multiplying the total nitrogen content with a conversion factor of 4.38 assuming 70% of nitrogen is proteinaceous (Chang & Miles, 1989) and expressed as percentage of sample DW.

$$\text{\% Total nitrogen content} = [(V \times M \times m)/wt] \times 100\%$$

$$\text{\% Crude protein content} = \text{\% total nitrogen content} \times 4.38$$

where V was the volume (ml) of HCl used; M was the molarity of HCl used; m was the molar mass of nitrogen and wt was the sample dry weight (g)

For residual ash determination, the Official Methods of Analysis (4.1.10) (AOAC, 1995) with some modifications was used. The other replicate of the sample residues were weighed into a pre-weighed porcelain crucible and combusted in a 550 ℃ muffle furnace overnight. After being cooled in a desiccator, the amount of the ash residue in the crucible was calculated by weight difference and expressed as percentage of sample DW.

2.3 Structural characterization of PSS and other commercial β-glucans

2.3.1 Monosaccharide profile study by gas chromatography (GC)

The following monosaccharide profile study was modified according to Pazur (1994).

2.3.1.1 Acid depolymerisation

Samples were subjected to acid hydrolysis for sugar composition determination. About 15 mg of sample was hydrolyzed with 0.7 ml 12M sulphuric acid at 35℃ for 1 h with stirring. The hydrolysate was diluted to 2M with 3.5ml of distilled water and heated in a boiling water-bath with shaking for 1 h. The hydrolysate was cooled to room temperature.

2.3.1.2 Neutral and amino sugar derivatization

Three milli-liters of hydrolysate was used to prepare alditol acetates of the neutral and amino sugars according to the method described by Blakeney *et al.* (1983). Beta-D-allose (1mg/ml) was added as an internal standard into sugar hydrolysate. Concentrated ammonia (12M) was then added to neutralize the hydrolysate followed by 5μl octan-l-ol to prevent foaming. Freshly prepared sodium borontetrahydride (200 mg/ml; 0.2 ml) was added to the neutralized hydrolysate and the mixture was kept at 40℃ for 30 min. The reduction by borontetrahydride was stopped by adding 0.1ml glacial acetic acid. Acetic acid anhydride (2.0 ml) was then added together with a catalyst, 1-methylimidazole (0.3 ml) and the acetylation was

proceeded at room temperature for 10 min. Distilled water (5.0ml) was added to decompose the excess acetic acid anhydride. Dichloromethane (1.0 ml) was added and the mixture was vortex-mixed, allowed to stand for 10 min at room temperature for phase separation. While the top aqueous layer was removed, the bottom organic layer was washed with distilled water (2 ml) twice and dried with anhydrous sodium sulphate and stored in a vial at -20℃ before GC analysis.

2.3.1.3 Determination of neutral sugars by gas chromatography (GC)

Alditol acetates of the neutral sugars were quantified by an HP6890 gas chromatography using an Alltech DB-225 capillary column (15m x 0.25mm i.d., 0.25 um film thickness) with the following oven temperature program: initial temperature, 180℃ with 4℃/min rise to 220℃ and held at 220℃ for 30 min. The injection sample volume was 2μl, the carrier gas was helium, and detection was done by flame ionization. Individual sugars were corrected for losses during hydrolysis and derivatization and for the response of the GC detector. The values for monosaccharides were expressed as polysaccharide residues (anhydro-sugars) by multiplying the amounts of pentoses and deoxypentoses by a factor of 0.88 and of hexoses by factor of 0.90.

2.3.2 Structural study of polysaccharides by methylation

2.3.2.1 Preparation of dry dimethyl sulfoxide (DMSO)

The methylation procedure was based on the method described by Harris *et al.* (1984) as well as Mukerjea *et al.* (1996). About 400 ml reagent grade DMSO was transferred with a glass syringe into a 500-ml round bottom flask, which was previously flushed with nitrogen and filled with pre-dried 4A molecular sieve. The flask was sealed tightly with a rubber cap and covered with aluminum foil to avoid the exposure to light, and finally was stored at 4℃ or room temperature.

2.3.2.2 Preparation of methylsulfinyl methyl sodium ($CH_3SOCH_2^-Na^+$) from the dry DMSO and sodium hydride

A total of ten grams of sodium hydride (NaH) (Sigma-Aldrich), weighed together with 5% mineral oil, were added into two separate 50-m centrifuge tube and hexane was filled up to a total volume of 30 ml. After centrifugation at 3000 rpm for 5 min, hexane was discarded. This hexane washing process was repeated to remove the oil on the surface of sodium hydride. Sodium hydride was consequently collected as a pellet in the centrifuge tube after six rounds of washing and transferred into a dried 3-necked flask previously flushed with nitrogen with a minimum amount of hexane. The 3-necked flask was flushed with argon to evaporate the remaining hexane. When the sodium hydride was dry, 30 ml of dry DMSO was added by a glass syringe into the 3-necked flask. The flask was kept at room temperature for overnight until the color changed to greylish green. The methylsulfinyl methyl sodium was then stored in several 2ml-vials, pre-flushed with nitrogen, and sealed by cap with

septum. Methylsulfinyl methyl sodium was stored away from light at -20℃. For methylation, methylsulfinyl methyl sodium was thawed and removed through the septum of the cap with the use of a glass syringe.

2.3.2.3 Methylation procedure

About 4 mg of PSS and other commercial β-glucans were weighed into a capped serum bottle and was dried with phosphorus pentoxide overnight in vacuum. After drying, 1 ml dry DMSO was added to the bottles with a glass syringe under argon. The mixture was vortex-mixed and sonicated at room temperature for 30-50 min.

When the samples were solubilized in DMSO, then 0.4 ml methylsulfinyl methyl sodium was added and the mixture was vortex-mixed. The gel formed after mixing was broken up by sonication at 25℃ for 60-90 minutes. Followed by the addition of 0.3 ml chilled methyl iodide, the mixture was sonicated at 25℃ for 15-20 min, and was kept at room temperature for 2 hours.

If the samples were insoluble in DMSO, premethylation was conducted to enhance solubility. Methylsulfinyl methyl sodium (20μl) was added and the mixture was kept in an ice bath until frozen; and a further 5 μl methyl iodide was added. The above 2 steps were repeated but with 60 μl methylsulfinyl methyl sodium and 15 μl methyl iodide. After premethylation, the samples were processed in the same way as the DMSO-soluble samples.

After 2h incubation, 0.3 ml distilled water was added to neutralize excess

methylsulfinyl methyl sodium and the resulting suspension was transferred into a 15-ml centrifuge tube. The methylated polysaccharides were extracted from the suspension with an addition of 4 ml chloroform, and were then washed by 3 ml distilled water. The mixture was centrifuged at 3000 rpm for 5 min and the top water layer was discarded after phase separation. This washing process was repeated 3 times. After the last wash, the remaining water in the tube was dried by adding 3 ml 2,2-dimethoxypropane and 40 μl glacial acetic acid. The solvent in the end product was removed by evaporation at 40℃ under nitrogen to give a dry product.

2.3.2.4 Preparation of partially methylated alditol acetates (PMAAs)

The methylated polysaccharide was hydrolyzed with 0.6 ml of 2M trifluoroacetic acid in a capped tube previously flushed with nitrogen and was heated at 121℃ for 1 hours. After the completion of hydrolysis, the tube was cooled to 40℃ and the hydrolysate was dried with nitrogen.

Then the hydrolysate of the methylated polysaccharide was reduced and acetylated by following the same procedure in the GC analysis, described in section 2.3.1.2, starting from the adjustment of pH by adding 0.5 ml of 12 M ammonia into the samples.

2.3.2.5 Analysis of the PMAAs by GC-MS

The procedure below was modified according to Sweet *et al.* (1975). The partially methylated alditol acetates (PMAAs) samples were analyzed by GC-MS

(GCMS-QP5050, Shimadzu, Japan). The GC part was equipped with an HP-5MS capillary column (5% phenyl methyl siloxane, 30 m x 25 μm i.d., Hewlett-Packard). Helium was the carrier gas and the flow rate was adjusted to 1.2 ml/min. The initial oven temperature was 130℃, and was increased to 280℃ at a rate of 3℃/min. The interface temperature was 250℃, and the split ratio was set at 30:1.

Regarding the MS conditions, the ion source temperature was 250℃ with an ionization energy of 70eV; the voltage of detector was 1.5 kV and mass range of 50-350. The spectra of the PMAAs were compared with that of known standards in the GC-MS library .

2.3.3 Intrinsic viscosity determination

The method was modified according to Wang *et al.* (2004). The viscosity of dilute PSS and commercial β-glucan solutions were measured using Ubbelohde viscometers (Cannon, #50 for PSS, PAC and CUR; #25 for LAM) fully submerged in a 37℃ water-bath.

About 40mg sample (100mg in the case of LAM) was dissolved in 10ml NaOH (1M) with mechanical stirring and the solution was filtered (0.45μm PVDF membrane filters, Millipore). This was used as the stock solutions.

Viscosity data at different concentrations of the samples were obtained by dilution of the above stock solution with NaOH (1M) to give concentrations of 0.4 g/100ml, 0.3g/100ml, 0.26g/100ml, 0.23g/100ml, 0.2g/100ml and 0.18g/100ml. (In the case of

LAM, viscosity data was collected at concentrations of 1g/100ml, 0.75g/100ml, 0.64g/100ml, 0.56/100ml, 0.5g/100ml and 0.45g/100ml).

Capillary viscometry is conceptually simple: the imte it takes a volume of polymer solution to flow through a thin capillary is compared to the time for a solvent flow (1M NaOH in this case). It turns out that the flow time for either is proportional to the viscosity, and inversely proportional to the density.

$$t_{solvent} = \eta_{solvent}/\rho_{solvent}$$

$$t_{polymer\ solution} = \eta_{polymer\ solution}/\rho_{polymer\ solution}$$

Relative viscosity [η_{rel}] is defined as $\eta_{polymer\ solution}/\eta_{solvent}$, while for most polymer solutions at the concentrations of interest, $\rho_{polymer\ solution}/\rho_{solvent} \approx 1$. Thus, to a very good approximation, the relative viscosity [η_{rel}] is a simple time ratio:

$$\eta_{rel} = t_{polymer\ solution}/t_{solvent}$$

The specific viscosity [η_{sp}] is also defined to be the fractional change in viscosity upon addition of polymer,

$$\eta_{sp} = (\text{Flow rate of the sample solution}/\text{ Flow rate of solvent}) - 1$$

Both the relative viscosity [η_{rel}] and specific viscosity [η_{sp}] depends on the polymer concentration, the intrinsic viscosity of a particular polymer is determined by extrapolating to zero concentration. Measuring at zero concentration is

meaningless, but the concept of extrapolating to zero concentration ($c \rightarrow 0$) was very important in polymer characterization. The two quantities that are commonly plotted vs concentration and extrapolated to c=0 are $c^{-1}\eta_{sp}$ and $c^{-1}ln\ (\eta_{rel})$.The two plots would theoretically have the same intercept, which was the intrinsic viscosity [η]. The the intrinsic viscosity [η] was found to be proportional to the molecular weight of the polymer.

2.4 Enzymatic digestion of PSS

2.4.1 Optimization of digestion Conditions

After the structural analysis of PSS, endo-beta-(1→ 3)-glucanase (EC 3.2.1.39) from *Trichoderma sp.* (Megazyme International, Ireland) was chosen for enzymatic digestion. One gram of PSS powder was hydrolyzed by 0.2 ml (160 units/ml) of endo-beta-(1 → 3)-glucanase in sodium citric buffer (100 mM, pH 4.5) at 40℃. The reaction mixture was sampled every hour for 24 times, and the aliquots (1 ml) were analyzed by HPLC. After 24h, the whole mixture was heated in a 90℃ water bath for 10 min to terminate the enzyme activity. The resulted oligosaccharide profile was monitored by an HP 1100 system using a BioRad Aminex® Carbohydrate HPX 42A column (300 mm x 7.8 mm) with degassed HPLC grade deionized water the mobile phase. The flow rate used was 0.4 ml/ min and the temperature was kept at 80℃. Each run lasted for 30 min. The injection sample volume was 70μl and signal was monitored by a Waters 410 Differential Refractometer, coupled with a signal interface (HP Interface 35900E). Optimal condition of enzyme digestion time interval of PSS was set to be the one that gave the largest production of oligosaccharides with degree of polymerization (DP) 2 to 10, which was estimated using laminarian standard (Megazyme International, Ireland).

2.4.2 Large scale oligosaccharide preparation by preparative HPLC

Glucose-based oligosaccharides with DP 2 to 10 were purified from PSS with a preparative HPLC system after enzymatic digestion with the optimal digestion

condition determined in 2.4.1. Five hundred grams of BioGel P-2 Gel fine (BioRad 150 – 4115), which could be used to fractionate molecules of molecular weight 100 to 1,800 (~ DP 2 to 10) was used for the purification. Temperature of the column was kept at 55℃ with a heat circulating water-bath. The flow rate was maintained at 1.2 ml/min by a peristaltic pump (Phamacia Pump P-50), and GradiFrac Fraction Collector (Pharmacia) was used for eluent collection. Ten millimeters of eluent was collected in each pre-cleaned test-tube, and detection was done by phenol-sulphuric acid test (for details, see 2.2.2) with 0.2 ml sampling volume from each test tube. Test tubes containing oligosaccharides of DP from 2 to 10 were pooled together, lyophilized and regarded as *Poria cocos* sclerotium oligosaccharide (PCO).

2.5 *In vitro* fermentation of β-glucans

2.5.1 Static Batch culture *in vitro* fermentation using human fecal inoculum

In order to assess the *in vitro* fermentability, PSS, PCO, other commercial β-glucans, the positive control FOS as well as the negative control cellulose were subjected to *in vitro* fermentation using a human fecal homogenate under strictly anaerobic conditions in a batch system for 24h as described by Bourquin *et al.* (1996) as well as Velázquez *et al.* (2000) with some modifications. Fresh human feces were separately collected from three healthy adult volunteers, who had consumed a non-specific Western/ Chinese mixed diet and had not taken antibiotics for the past three months with no history of gastrointestinal diseases prior to the study. In brief, individual fresh fecal sample was immediately collected into an autoclaved and pre-weighed 100 ml reagent bottle containing 49 ml of 100 mM reduced sodium phosphate buffer (pH 6.8, Table 2.1) as well as 1 ml of oxygen reducing enzyme supplement (Oxyrase® For Broth, Mansfield, OH, USA), which when used together could generate and maintain the internal anaerobic condition within the sampling bottles. The sampling bottles were then flushed with ultra-pure argon, tightly closed and sealed with parafilm before the preparation of faecal inoculum. As soon as possible, the three faecal samples obtained were aseptically pooled together and homogenized for 50sec in a Waring blender at low speed under anaerobic condition provided and maintained by the mixture of reduced sodium phosphate buffer and the oxygen reducing enzyme supplement as well as continuous flushing of ultra-pure argon. The resulting human faecal homogenate was then further diluted with the reduced sodium phosphate buffer (containing Oxyrase® For Broth, 50:1 v/v) to a

final ratio of 1:4 (w/v) and passed through three layers of autoclave-sterilized filtering bags. The filtrate of this diluted human faecal homogenate was immediately used as the fermentation inoculum.

Triplicate samples (500 mg) of the substrates, the control (FOS) and cellulose were separately and aseptically added into autoclave-sterilized 100 ml reagent bottles containing 40 ml of the reduced sodium phosphate buffer. The bottles were then tightly closed, sealed with parafilm and stored at 4℃ for 16 - 24 h in order to allow complete hydration of the substrates and to limit the possibility of microbial growth before initiating fermentation. Two hours before inoculation, 0.8 ml of the oxygen reducing enzyme supplement was aseptically applied into the substrate mixture and the headspace of the bottle was flushed with ultra-pure argon gas for 1 min. The bottles were then tightly closed, sealed with parafilm and pre-incubated at 37℃. To begin fermentation, each substrate-containing bottle was aseptically inoculated with 10 ml of the aforesaid human faecal homogenate and the headspace of the bottle was flushed with the ultra-pure argon gas for 1 min. All bottles were then tightly closed, sealed with parafilm and incubated for 24 h with mild agitation (50 rpm) on a vibration shaker at 37℃. One ml and 0.375 ml aliquots were taken out from each sample bottle at 0 h, 6 h, 12 h and 24 h for short chain fatty acids analysis (refer to 2.6) and microbial analysis by fluorescent *in situ* hybridization (FISH) (refer to 2.7) respectively. At the end of the experiment, the non-fermented residues of the control and samples were individually well-mixed by vigorous shaking for pH measurements. These non-fermented residues were then subjected to organic matter disappearance (details refer to 2.5.2). Following the same procedure, triplicate of reagent bottles

containing no substrate (blank) were also fermented with the human faecal homogenate in order to allow corrections for any dry matter, organic matter and SCFAs production not arising from the substrates. These SCFA correction accounted for SCFAs originally present in the donor faeces, and SCFAs produced during fermentation of organic matter originally present in the inoculum as well as fermentation medium.

Table 2.1 Chemical composition of fermentation medium (reduced sodium phosphate buffer)* used for *in vitro* fermentation

Component	Concentration in medium
Sodium Phosphate buffer (100 mM, pH 6.8)	Per L
Na_2HPO_4	11.5 g
$NaH_2PO_4 \bullet 2H_2O$	3.1202 g
Reducing solution	Per 100 ml
Cysteine hydrochloride	0.625 g
$Na_2S \bullet 9H_2O$	0.625 g
1 M NaOH	4 ml

*To prepare reduced sodium phosphate buffer, 33.5 ml of the reduced solution was added to 1 L of the sodium phosphate buffer just before autoclave sterilization.

2.5.2 Determination of organic matter disappearance (OMD)

Organic matter contents of the non-fermented residues of the control and the other samples were determined according to the procedure described by Bourquin *et al.* (1996). In brief, individual non-fermented sample was combined with four volumes of 95% EtOH to precipitate the non-fermented residues overnight. After filtered through a tared Whatman 541 ashless filter paper, the residue recovered was

sequentially washed with 78% EtOH, 95% EtOH and acetone followed by oven-drying at 105℃ for 24 h. For FOS, PCO and LAM, the filtrates were further subjected to drying by SpeedVac Concentrator (Thermo Savant SPD 121 P-230) connected to a Refrigerated Vapor Trap (Thermo Savant SV-RVT 4104-230D) with the trap temperature at - 100°℃ to -110℃ as well as a Rotary Vane Oil Pump (Thermo Savant VLP 120 – 230) before overnight oven drying. This was to recover the unfermented substrates which were mainly of small molecular weight and could not be precipitated by 95% ethanol. Together with the ashless filter paper, each oven-dried residue was then quantitatively transferred to a pre-weighed and acid-washed porcelain crucible and dry ashed in a muffled furnace at 550℃ overnight. The ash content of the non-fermented residues was weighed for computing their organic matter recoveries. Following the same procedure, the organic matter of the original controls and samples (starting materials for the *in vitro* fermentation) were also analyzed.

The organic matter disappearance (OMD) was calculated as follows,

$$\text{Organic matter disappearance (\%)} = \frac{OM_{original} - (OM_{24} - B_{24})]}{OM_{original}} \times 100\ \%$$

where $OM_{original}$, is the weight of organic matter of the original β-glucan sample;

OM_{24} is the weight of organic matter of the non-fermented residue after 24h fermentation;

B_{24} is the average weight of organic matter in the blank bottles after 24 h fermentation.

2.6 Gas chromatographic determination of SCFAs

The method to measure SCFA concentration was modified from Erwin and his co-investigators (1961). Aliquots (1 ml) taken out at different incubation time during *in vitro* fermentation as aforementioned were transferred to a 1.5ml-eppendorf and frozen at -20 ℃ until SCFA analysis. Briefly, frozen aliquot was thawed and centrifuged (4800 rpm, 4℃, 30 min). Three hundred and fifty microlitres of the supernatant was then transferred into a new 1.5 ml eppendorf and acidified by adding 87.5 μl of 25% meta-phosphoric acid together with 62.5 μl of an internal standard, 4-methyl pentanoic acid (4 mg/ml of 25% meta-phosphoric acid), with vortex-mixing. After incubated at room temperature for 30 min, the mixture was vortex-mixed with 0.5 ml of diethyl ether (good organic solvent for extraction of SCFAs) and allowed to stand for 10 min for phase separation. The top layer (diethyl ether with dissolved SCFAs) was transferred into another 1.5ml-eppendorf and the bottom layer (containing remaining SCFAs) was further extracted two times with 0.5 ml of the diethyl ether in order to recover as much remaining SCFAs as possible. The SCFA extract were pooled together followed by dehydration with anhydrous sodium sulphate. The dehydrated SCFA sample was then transferred into a sample vial through a 0.45μm filtering disc and stored at –20℃ until GC analysis. The SCFA content of the controls and the studied samples produced after different *in vitro* fermentation incubation time were quantified by a HP 6890 GC system equipped with an Alltech Quadrex® 007-FFAP capillary column (30 m × 0.25 mm; i.d. 0.25 μm film), coupled with an FID detector. An oven temperature program of initial temperature 100℃ with a hold of 5 min, followed by a temperature rise of 3℃/min

to 160°C with a final hold of 5 min was used. Both injector and detector temperature were set at 220°C. Helium was used as carrier gas with a constant flow rate of 1.5 ml/min while compressed air and hydrogen were used as detector gases. Five microlitres of sample was injected with a split ratio of 20: 1 and their SCFA content were detected by flame ionization. Individual SCFAs was corrected for losses during solvent extraction as well as their different responses to the GC detector by a molar correction factor determined from the recovery of SCFA standards subject to the same sample treatment. A mixture of individual SCFA standards including ethanoic acid (i.e. acetic acid), propanoic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid (SCFA standards kit, catalogue no. 18600, Alltech, PA, USA) and 4 methyl-pentanoic acid (internal standard, catalogue no. 6220601, Alltech) was prepared in 25% meta-phosphoric acid at a final concentration of 0.5 mg/ml for both quantitation and identification. The amount of the SCFAs produced from the positive and negative control as well as the studied β-glucans after *in vitro* fermentation were expressed as millimoles per gram of substrate on organic matter basis.

The correction factors, CF_{SCFA}, for each SCFAs was calculated by using the following equation:

$$CF_{SCFA} = \frac{(P_{IS} \times W_{SCFA})}{(P_{SCFA} \times W_{IS})}$$

where P_{IS} = GC peak area for internal standard (4-methyl pentanoic acid);

W_{SCFA} = weight (mg) of individual SCFAs standard used in the assay;

P_{SCFA} = GC peak area for individual SCFAs standard;

W_{IS} = weight (mg) of the internal standard used in the assay.

The content (mmol/g of substrate on organic matter basis) of individual SCFA produced by the sclerotial TDF sample was computed as follows:

$$\textbf{Individual SCFA (mmol/g)} = \frac{(CF_{SCFA} \times P_{SCFA} \times W_{IS} \times S_{TOTAL} \times FR_{TOTAL})}{(P_{IS} \times S_{ASSAY} \times FR_{ASSAY} \times MW \times W_S)}$$

where CF_{SCFA} is correction factor for individual SCFA

P_{SCFA} is the GC peak area for individual SCFA in sample solution;

W_{IS} is the weight (mg) of internal standard in sample solution;

S_{TOTAL} is the total volume of supernatant from 1.0 ml of fermented residue and ranged from 0.5ml to 0.9ml in different samples;

FR_{TOTAL} is the total volume of fermented residue and is equal to 50 ml;

P_{IS} is the GC peak area for internal standard in sample solution;

S_{ASSAY} is the aliquot of supernatant for assay and is equal to 0.35 ml;

FR_{ASSAY} is the aliquot of fermented residue for assay and is equal to 1.0 ml;

MW is the molecular weight (g/mol) of individual SCFA (60.05 for ethanoic acid, 74.08 for propanoic acid, 88.11 for butanoic acid, 102.13 for pentanoic acid, 116.16 for hexanoic acid, 130.19 for heptanoic acid, 144.21 for octanoic acid);

W_S is the weight (organic matter, mg) of original sample.

2.7 Microbial identification and enumeration

2.7.1 Oligonucleotide probes for fluorescent *in situ* hybridization

Genus-specific 16S rRNA-targeted probes for enumerating total number of fecal bacteria and *Bifidobacterium*. (details shown in Table 2.2) were synthesized commercially and 5'-labelled with the fluorescent dye Cy 3 (Proligo, Paris, France). The samples were also counter-stained with the nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI) to obtain the total cell counts. Cells were fixed onto a glass slide, and those stained with DAPI or hybridized with probe were enumerated under a microscope, as described below.

Table 2.2 Oligonucleotide probes used for target bacteria enumeration

Target bacteria	Probe	Probe (5' to 3') sequence	Hy temp* (℃)	Reference
Total count	Bac 338	GCTGCCTCCCGTAGGAGT	54	Amann *et al.*, 1990
Bifidobacterium	Bif 164	CATCCGGCATTACCACCC	50	Langendijk *et al.*, 1995

*Hybridization temperature

2.7.2 Fluorescent *in situ* hybridization (FISH)

The procedures of FISH were modified according to the method described by Rycroft *et al.* (2001) as well as Ames *et al.* (1999).

2.7.2.1 Cell Fixation

Static batch culture fermentations were set up as described above (details refer to 2.5.1) and samples removed for bacterial enumeration via FISH. After 0h, 12h and 24h of fermentation, samples(375 µl) were removed from the batch cultures and added to 1.125ml of filtered 4% (w/v) paraformaldehyde solution (pH 7.2), vortex-mixed, and stored at 4℃ overnight for cell fixation. The fixed cells were centrifuged at 2500 rpm for 10 min and washed twice in filtered 0.1M phosphate buffer solution (0.1 M, pH 7.0). This was then resuspended in 150µl phosphate buffer solution (0.1M, pH 7.0). Ethanol (150 µl) was added, and the samples were vortex-mixed and stored at - 20℃ until further analysis.

2.7.2.2 *In situ* hybridization

The fixed cells (10µl) were added to 70µl pre-warmed HPLC grade water and 200 µl pre-warmed, filtered, double-strength hybridization buffer (1.8M NaCl, 40mM Tris HCl, pH 7.2, 0.2% SDS (w/v)), and mixed. For each hybridization, 45 µl fixed cells were added to each probe ($50mgl^{-1}$) in a ratio of 9:1 (v/v), mixed and placed in the appropriate hybridization temperature (for details, see Table 2.2) for 24 h. The hybridized sample (1 – 50 µl) was then washed in 5ml pre-warmed, filtered, single-strength hybridization buffer (0.9M NaCl, 20mM Tris-HCl, pH 7.2) containing 20 µl DAPI solution($500gl^{-1}$) for 30 min at the appropriate hybridization temperature (for details, see Table 2.2). The sample was then filtered onto a 0.2 µm pore size GTBP Isopore black membrane filter (Millipore Corporation, Watford, Herts., UK) and the filter mounted in Slow-fade (Molecular Probes, Leiden, The

Netherlands) onto a clean microscopic slide. Cells were counted using a Nikon Microphot EPI fluorescent microscope (Nikon, Kingston upon Thames, Surrey, UK) fitted with DM 400 and DM 510 filters (Nikon) for the DAPI stain (ex 359nm and em 461nm) and the Cy 3 dye (ex 550nm and em 565nm), respectively. A minimum of 15 fields, each containing 10 – 100 cells, was counted for each preparation. Cell counts for total number of fecal bacteria and *Bifidobacterium* at different incubation time during *in vitro* fermentation were expressed as cfu per feces wet weight.

2.8 Statistical analysis

Results were expressed as mean values ± standard deviation of their mean. Data were analysed using an one-way analysis of variance (ANOVA, Tukey) to determine the significance of mean differences between groups (SPSS 13.0 for windows). Differences were accepted as statistically significant at $p < 0.05$ level. Relationships between various parameters during 24h *in vitro* fermentation, including OMD, total, individual as well as overall SCFA production, total microbial count and bifidobacterial count made by FISH were analysed by Correlation (Pearson's test) (SPSS 13.0 for windows).

Chapter 3 Results and discussions

3.1 Chemical characterization of *Poria cocos* sclerotium

The amount of soluble protein, total sugar as well as the total dietary fiber content of PC sclerotium and PSS after freeze-drying was shown in table 3.1. Dietary fiber was the major component in natural PC sclerotium (>85%), with a small amount of soluble protein existed, as determined by the Lowry method (details refer to 2.2.1). Total sugar content as determined by the phenol-sulphuric acid test (details refer to 2.2.2) was comparable to that of the fiber content, implying that almost all the sugars determined were originated from dietary fiber, with only a small amount of simple sugar existed. After the extraction by sonication in an alkaline medium, dietary fiber (the NDC content) of PSS reached almost 95%, while the soluble protein content had almost been eliminated during the extraction.

Table 3.1 Soluble Protein, total sugar and total dietary fiber content (% dry weight)* of PC sclerotium and PSS after freeze-drying

	Soluble protein	Total Sugar Content	TDF
PC sclerotium	0.01 ± 0.00	88.82 ± 15.97	86.52±2.11
PSS	0.00 ± 0.00	98.38 ± 3.19	94.48±4.13

*Data are presented as mean % dry weight ± S. D., n=2.

3.2 Structural characterization of PSS & other commercial β-glucans

3.2.1 Monosaccharide profile

The monosaccharide profiles of the original PC sclerotium and the sonicated fraction PSS are shown in Table 3.2. Other commercial polysaccharides (PAC, CUR and LAM) were also subjected to GC analysis for confirmation of their monosaccharide profiles. The major monosaccharide constituent of PC sclerotium was glucose, which was up to 97% of PC sclerotium polysaccharide (Table 3.2). Rhamnose, galactose, arabinose, mannose, glucosamine and ribose also existed, but in a much smaller amount. Upon extraction with sonication in an alkaline medium, the resulting fraction PSS contained nearly 100% glucose (Table 3.2).

As given by the manufacturer, the major monosaccharide constituent in all the tested commercial β-glucans is predominantly glucose. In the cases of PAC and CUR, only very little amount of other monosaccharides were present (less than 2.5% and 0.5% respectively), with percentage of glucose nearly 100% (Table 3.2). However, in the case of LAM, other monosaccharides like fucose, mannose and galactose were present in substantial amounts (4.19%, 2.90% and 1.11%, respectively). As LAM was extracted from seaweed, other algal polysaccharides like fucan and alginic acid also existed. The 'fucan' contained varying proportions of fucose, galactose and glucuronic acid, small amounts of xylose, mannose, glucose, half ester sulphate and protein (Finch *et al.*, 1986). Therefore, the purity of β-glucans in LAM might not be as high as the others, yet, glucose still accounted for more than 90% of carbohydrates in this polysaccharide.

Table 3.2 Monosaccharide composition (%)* of PC sclerotium, PSS and other commercial β-glucans determined by Gas Chromatography (GC)

β-glucan	Rha	Fuc	Rib	Ara	Xyl	Man	Gal	**Glc**	Glc-NAc	Gal-NAc
PC	0.67	0.00	0.17	0.55	0.00	0.51	0.61	**97.20**	0.30	0.00
PSS	0.50	0.00	0.16	0.00	0.00	0.12	0.30	**98.92**	0.00	0.00
PAC	0.52	0.00	0.19	0.08	0.00	0.31	0.24	**97.68**	0.00	0.97
CUR	0.42	0.00	0.00	0.07	0.00	0.00	0.00	**99.50**	0.00	0.00
LAM	0.51	4.19	0.00	0.07	0.32	2.90	1.11	**90.90**	0.00	0.00

*Data are average value of duplicates and expressed as percentage of the total sugar determined.

**Rha: Rhamnose; Fuc: Fucose; Rib: Ribose; Ara: Arabinose; Xyl: xylose; Man: Mannose; Gal: Galactose; Glc: Glucose; Glc-NAc: glucosamine; Gal-NAc: galactoglucosamine

3.2.2 Glycosidic linkages in polysaccharides

Gas chromatography (GC) (details refer to 3.2.1) revealed that the major monosaccharide component in PSS and other commercial β-glucans was glucose. The types of glycosidic linkages were analyzed by gas chromatography-mass spectrophotometry (GC-MS). With the condition mentioned in session 2.3.2.5, the internal standard allose showed a sharp peak at time 29.80min (data not shown), which was identified as D-glucitol, hexaacetate (#8192). Except the internal standard peak, the chromatograms of PSS, PAC and CUR showed a single sharp peak at time around 19.2 min (data not shown). After comparing with the database, the peak was identified as 2, 4, 6-tri-o-methyl glucose (#51901), representing a 1→3 glycosidic linkage (During methylation, all the carbon molecules of the hexoses on the polysaccharides, except carbon number five and those that formed linkages with another carbon molecule would be methylated. The polysaccharides would then be completely hydrolysed to the corresponding monosaccharides, where the carbon molecules, formerly forming linkages with another carbon molecule and thus non-methylated, would be acetylated. Therefore, 2, 4, 6-tri-o-methyl glucose indicated a chain of glucose with carbon number 2, 4 and 6 unlinked, showing a 1→3 glycosidic linkage). In the case of LAM, a small peak (2.29% of total peak area detected) at time 26.26min (data not shown), which was identified as 2, 3, 4-tri-o-methyl glucose (#51907), showing a probability that there might be occasional 1 →6 glucose linkages branching on the main 1→3 glucan backbone (31.34% of total peak area detected).

Table 3.3 shows the proportion of 1→3 and 1→6 linkages in the β-glucans

investigated. It should be noted that the ratio of 1→3/ 1→6 linkages to terminal did not necessarily reflect the polysaccharide chain-length of the β-glucans, probably due to the different reaction of β-glucans towards the same methylation process (details refer to 2.3.2.3), but it did serve as a good indicator of the types of linkages present in the sample. All PSS, PAC and CUR had a linear 1→3 glycosidic linkages with little branches and terminal residues, indicating their relative long chain-length. LAM, on the other hand, was found to have one 1→6 branching in approximately every 20 glucose residues on the main 1→3 backbone (Table 3.3)

Table 3.3 Glycosidic linkages (%)* of various β-glucans determined by gas chromatography-mass spectrometry (GC-MS)

	1→3	1→6	Terminal
PSS	47.62%	Not detected	Not detected
PAC	38.85 %	Not detected	0.26%
CUR	45.03%	Not detected	0.81%
LAM	31.54%	1.73%	2.29%

*Data are presented as percentage (%) of total area detected in each chromatograph.

3.2.3 Molecular weight comparison as determined by intrinsic viscosity

Intrinsic viscosity [η] is one of the most common ways for determining the average molecular weight of polymers. As polysaccharides originated from different areas or growth conditions might possess different physical or chemical properties, in this experiment, the intrinsic viscosity [η] of PSS and several commercial glucans were determined and these were compared to the published molecular weight values. As both the relative viscosity [η_{rel}] and specific viscosity [η_{sp}] (definitions see 2.3.3)

depends on the polymer concentration, the intrinsic viscosity of a particular polymer is determined by extrapolating to zero concentration. Measuring at zero concentration is meaningless, but the concept of extrapolating to zero concentration ($c \rightarrow 0$) was very important in polymer characterization. The two quantities that are commonly plotted vs concentration and extrapolated to c=0 are η_{sp} and $c^{-1}ln$ (η_{rel}).The two plots would theoretically have the same intercept, which was the intrinsic viscosity [η]. Plots of η_{sp} and $c^{-1}ln$ (η_{rel}) of PSS, PAC, CUR and LAM are shown in Fig.3.1 to Fig 3.4, respectively. The intrinsic viscosity [η] of the tested β-glucans and a comparison of their reference molecular weight values were shown in Table 3.4.

Table 3.4 Intrinsic viscosity [η] of PSS and commercial β-glucans

	Intrinsic viscosity [η] (dL/g)	Reference Value of Mw*	Mw Reference
PSS	0.89	N/A**	N/A
PAC	0.89	$2.06 \times 10^4 - 8.93 \times 10^4$	(Zhang *et al.*, 1997)
CUR	3.53	$5.3 \times 10^4 - 2.0 \times 10^6$	(Nakata *et al.*, 1998)
LAM	0.02	7.70×10^3	(Mueller *et al.*, 2000)

*Mw: Molecular weight

** N/A: No published data available

The intrinsic viscosity [η] of CUR was the highest among the β-glucans tested. This matched with the reference values that curdlan generally possessed the largest molecular weight, from 5.3×10^4 to 2.0×10^6. As expected, [η] of PSS was exactly the same as that of the commercial PAC, which was also extracted from PC. LAM extracted from algae was found to have the lowest intrinsic viscosity [η] among these different sources of β-glucans.

Owing to the limited amounts of PCO prepared from preparative HPLC (details refer to 3.3.2), the molecular weight of PCO was not determined by intrinsic viscosity but estimated directly from the profile revealed by HPLC. The molecular weight of PCO (DP 2 – 10) should be 360 to 1800, using 180 as the reference molecular weight of one glucose molecule. Therefore, molecular weights of the β-glucans in descending order were as follows:

$$CUR > PAC \approx PSS > LAM > PCO$$

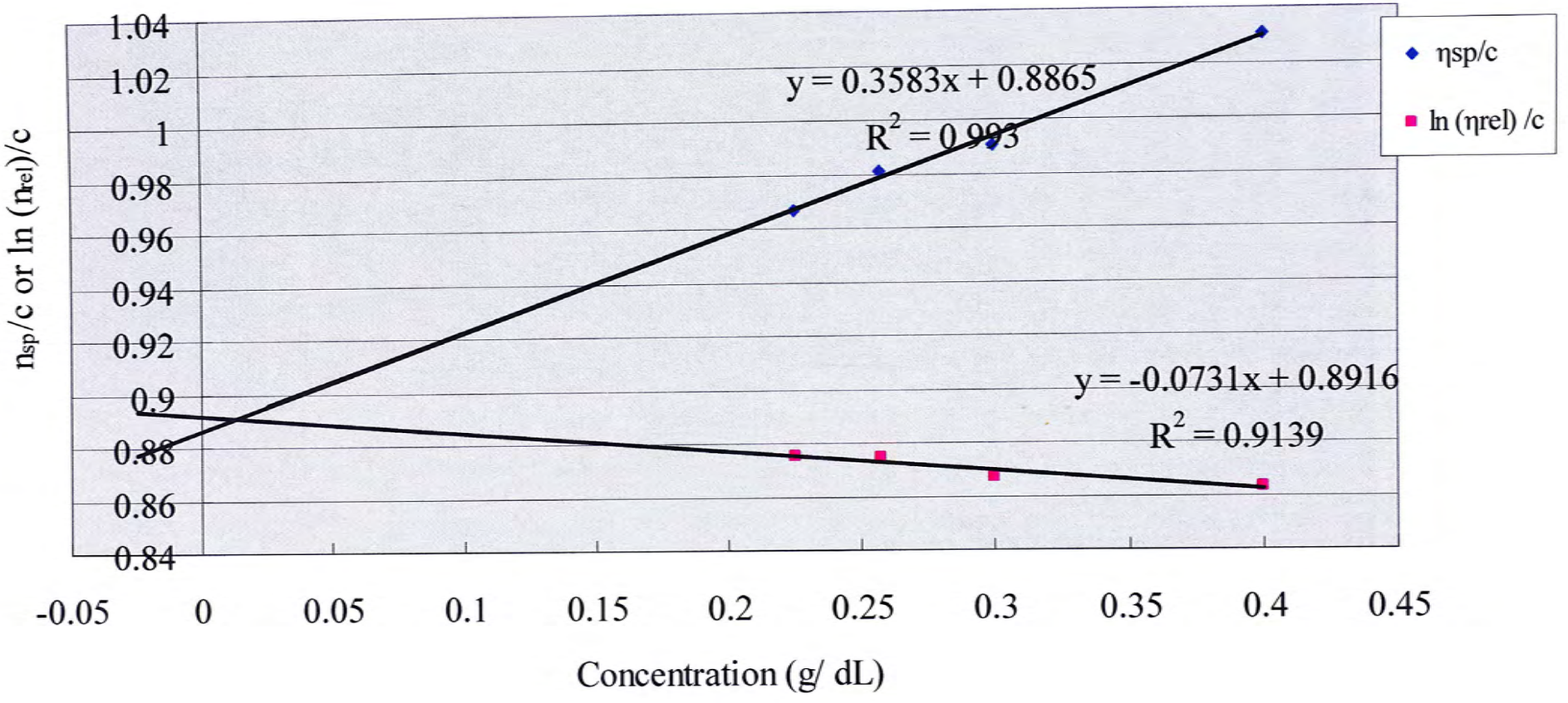

Fig 3.1 Determination of intrinsic viscosity [η] of PSS. The specific viscosity η_{sp} and the relative viscosity η_{rel} were determined by a Ubbelohde capillary viscometer at 37℃. Intrinsic viscosity [η] was defined as the common intercept of the two plots η_{sp}/c and ln (η_{rel})/c, which had been extrapolated to c = 0 (n=3).

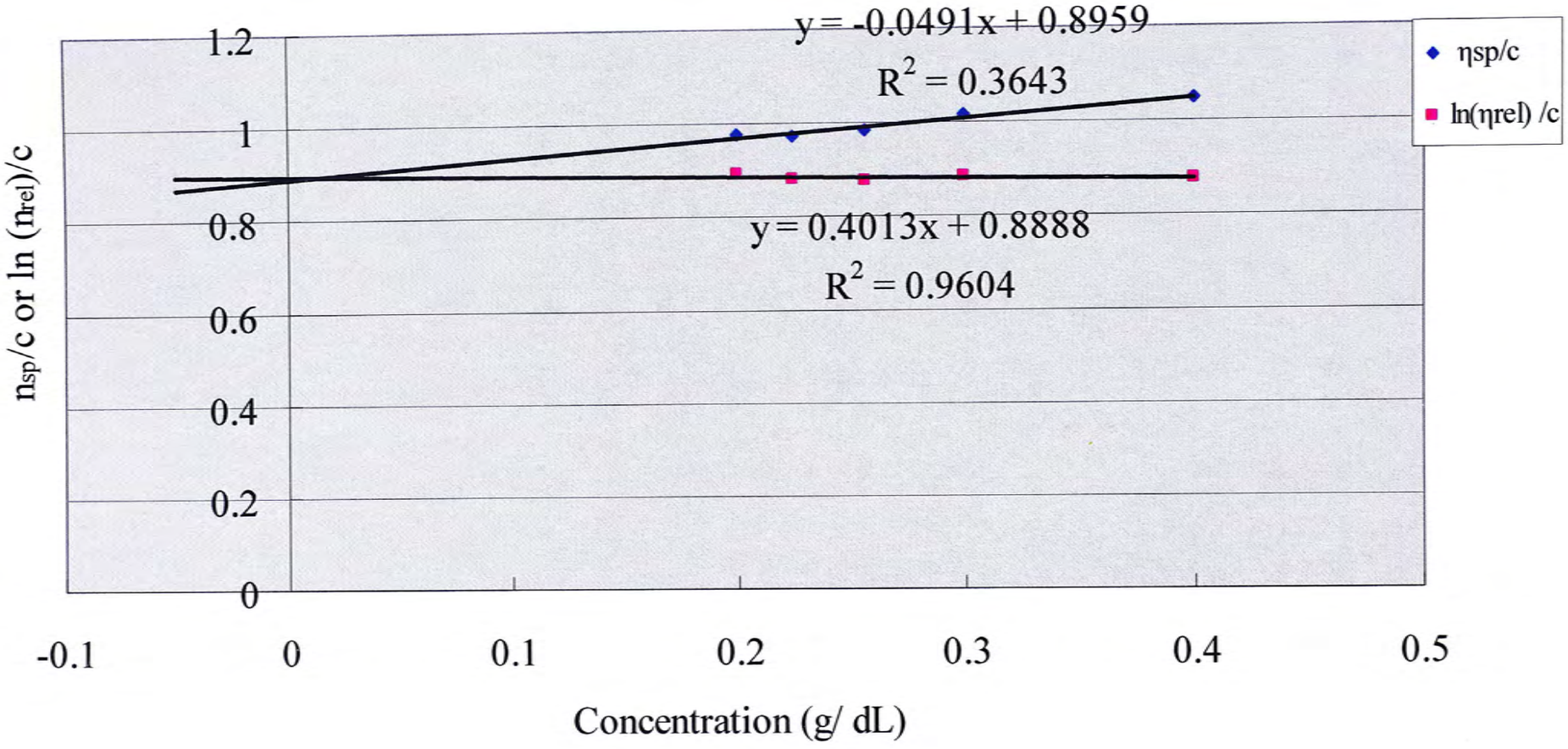

Fig 3.2 Determination of intrinsic viscosity [η] of PAC. The specific viscosity η_{sp} and the relative viscosity η_{rel} were determined by a Ubbelohde capillary viscometer at 37℃. Intrinsic viscosity [η] was defined as the common intercept of the two plots η_{sp}/c and ln $(\eta_{rel})/c$, which had been extrapolated to c = 0 (n=3).

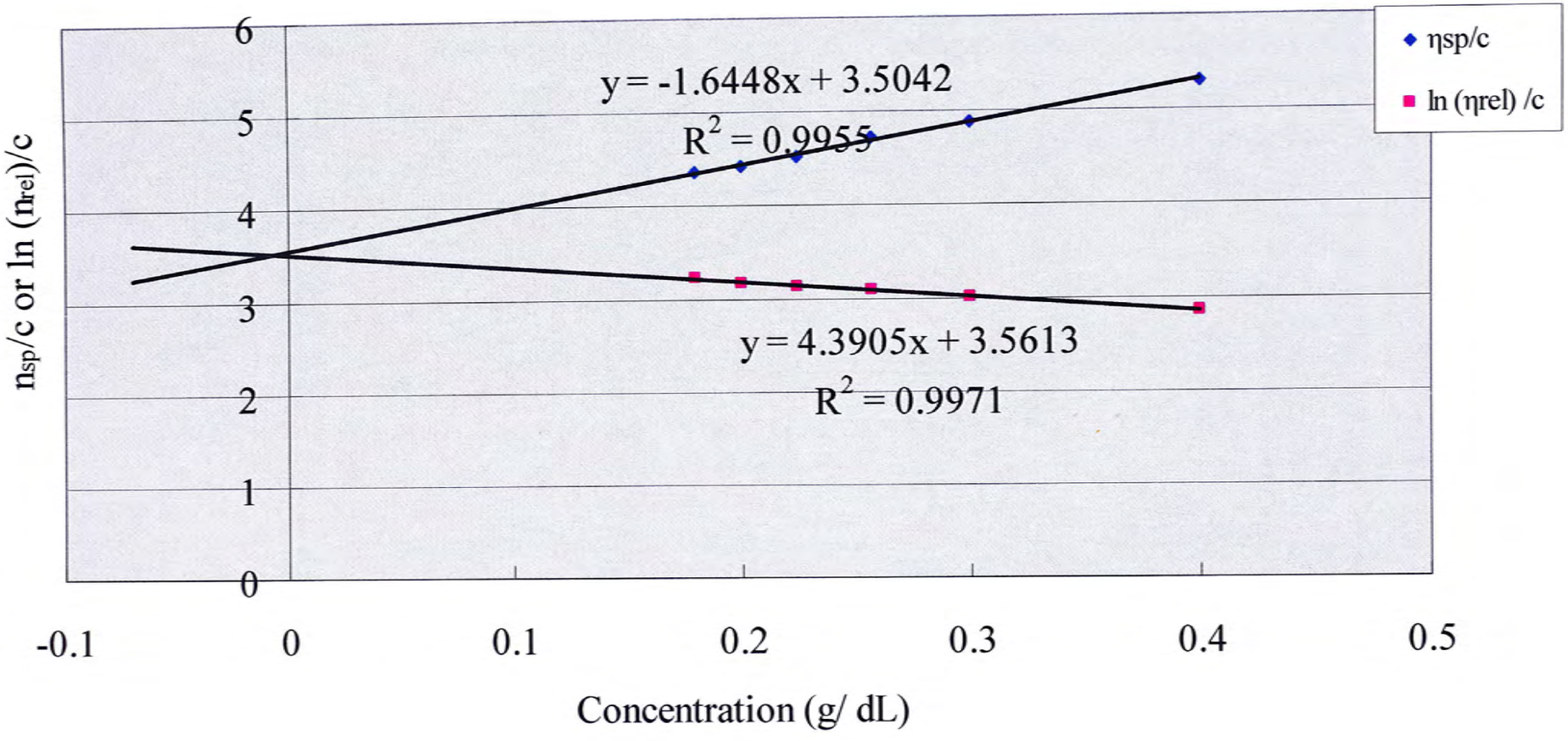

Fig 3.3 Determination of intrinsic viscosity [η] of CUR. The specific viscosity η_{sp} and the relative viscosity η_{rel} were determined by a Ubbelohde capillary viscometer at 37℃. Intrinsic viscosity [η] was defined as the common intercept of the two plots η_{sp}/c and ln (η_{rel})/c, which had been extrapolated to c = 0 (n=3).

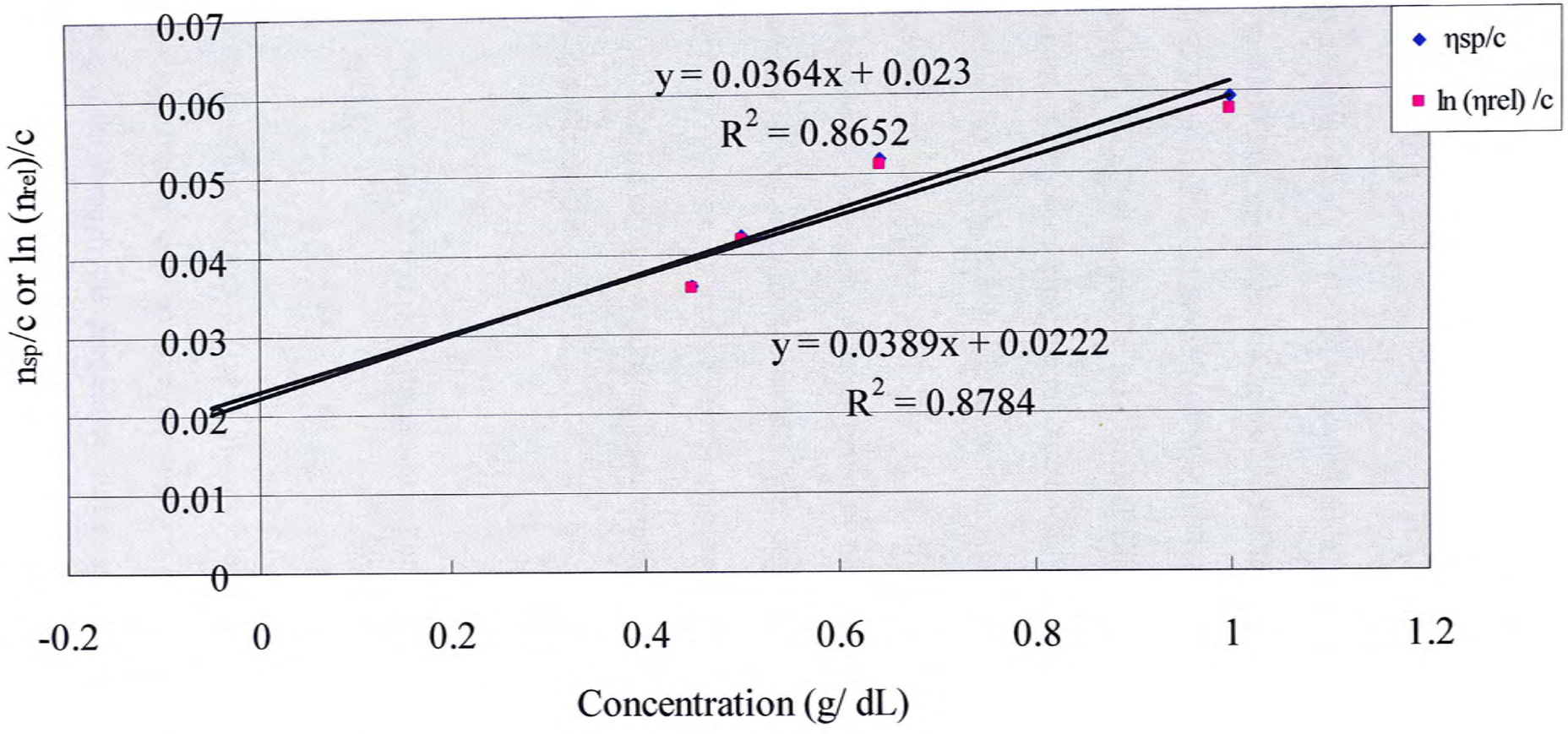

Fig 3.4 Determination of intrinsic viscosity [η] of LAM. The specific viscosity η_{sp} and the relative viscosity η_{rel} were determined by a Ubbelohde capillary viscometer at 37℃. Intrinsic viscosity [η] was defined as the common intercept of the two plots η_{sp}/c and $\ln(\eta_{rel})/c$, which had been extrapolated to c = 0 (n=3).

3.3 Preparation of β (1→3) glucose-based oligosaccharides

3.3.1 Enzymatic digestion of PSS

In order to obtain β (1→3) oligosaccharides, endo-β-(1→3)-glucanase was used to hydrolyze the glycosidic linkages of PSS. Oligosaccharide concentration was determined by summation of the amount of gluco-oligosaccharides of DP 2 to 10 as determined by HPLC at a specific time interval during enzyme digestion, and was expressed as mg oligosaccharide per ml digestion buffer. (details refer to 2.4.1). The results are shown in Fig. 3.5. It was shown that the oligosaccharide (DP 2 to 10) concentraton was the highest after four hours of digestion, and the amount of oligosaccharides in the digestion buffer gradually declined afterwards, as more and more polysaccharides and oligosaccharides were converted to their monosaccharide constituent, i.e. glucose, by endo-β-(1→3)-glucanase. Therefore, the 4h digestion mixture was selected to prepare purified β-glucose-based oligosaccharide, by using a preparative HPLC system.

3.3.2 Preparation of β (1→3) glucose-based oligosaccharides by preparative HPLC

In order to collect enough β (1→3) glucose-based oligosaccharides for *in vitro* fermentation, a total of eight enzyme digestion was carried out and the mixture was allowed to pass through the preparative HPLC system to remove glucose and polysaccharides with DP > 10. Enzyme digestate (4h) of PSS (1g) by endo-β-(1→3) glucanase at 40℃, in a citric buffer (100nM, pH 4.5) was lyophilized and dissolved

again in 10 ml HPLC grade water before preparative HPLC injection. The eluent was collected in test tubes for sugar content determination by phenol-suphuric acid test. A total of 170 test tubes, each of 10 ml eluent were collected.

The oligosaccharide profile of the preparative HPLC system using a 4h digestion mixture is shown in Fig 3.6. As shown, the oligosaccharides produced were mainly of DP 2 to 4, but those of DP 5 to 10 were also collected. The yield of each collection varied, but was generally between 200 to 300 mg. The oligosaccharides were then lyophilized for water removal, and the fraction PCO were then used for batch culture *in vitro* fermentation for prebiotic potential investigation.

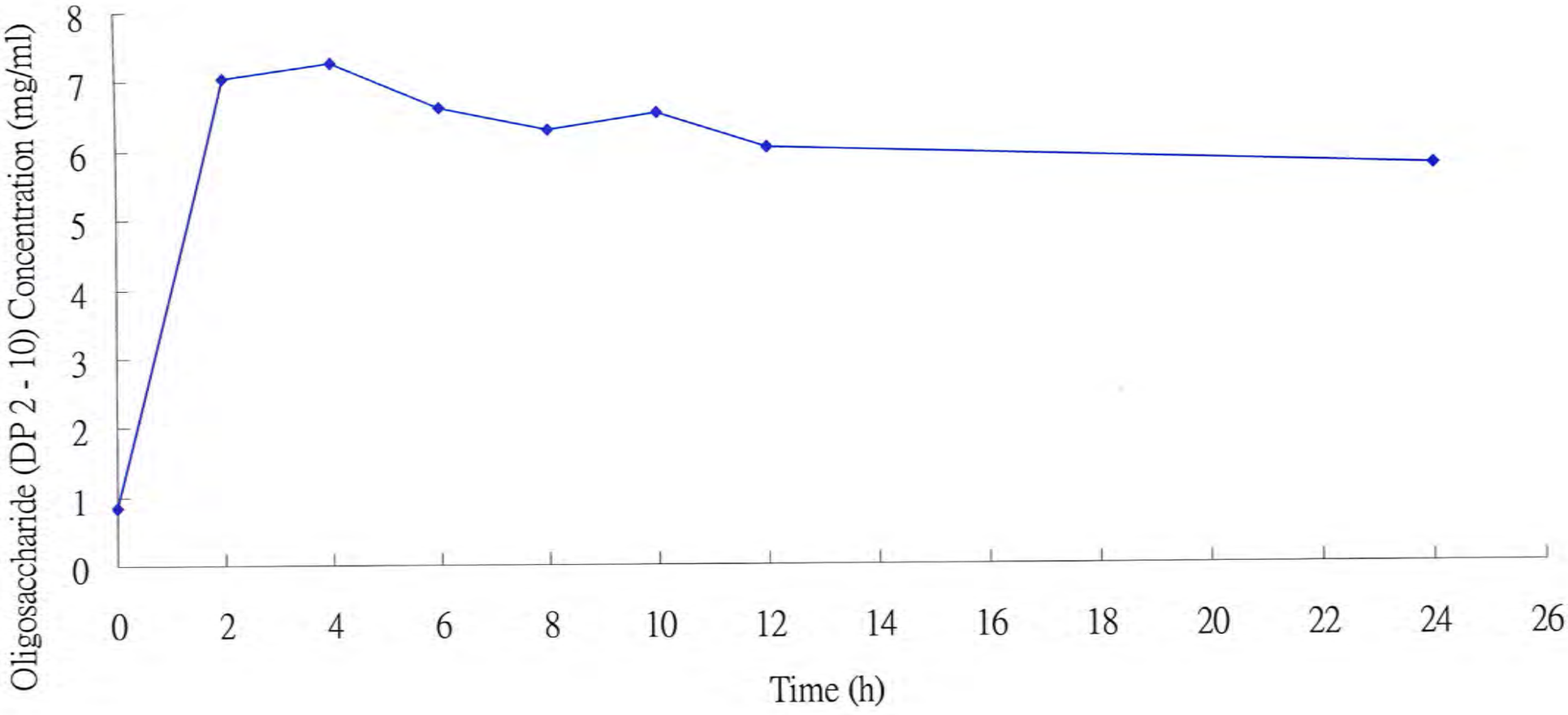

Fig 3.5 Oligosaccharide concentration (mg/ml digestion buffer) with DP 2 to 10 upon 24h hydrolysis of PSS (1g) by endo-β-1→3 glucanase at 40℃, in a citric buffer, pH 4.5. Oligosaccharide concentration was determined by summation of the amount of gluco-oligosaccharides of DP 2 to 10 as determined by HPLC at a specific time interval during enzyme digestion, and was expressed as mg oligosaccharide per ml digestion buffer.

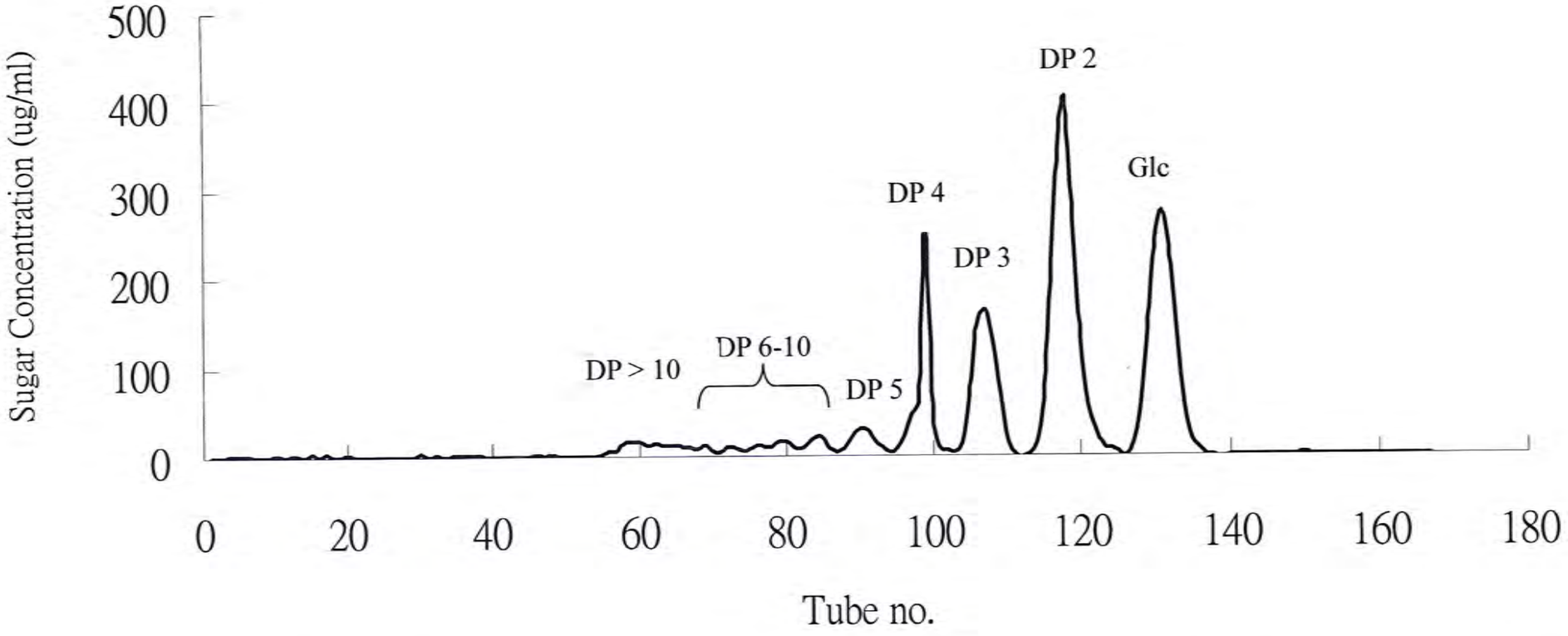

Fig 3.6 Sugar content as determined by phenol-suphuric acid test of eluent of preparative HPLC using degassed HPLC grade water as the carrier solvent. Enzyme digestate (4h) of PSS (1g) by endo-β-(1→3) glucanase at 40℃, in a citric buffer (100nM, pH 4.5) was lyophilized and dissolved again in 10 ml HPLC grade water before preparative HPLC injection. The eluent was collected in test tubes for sugar content determination by phenol-suphuric acid test. A total of 170 test tubes, each of 10 ml eluent were collected.

3.4 Batch culture *in vitro* fermentation

3.4.1 Organic matter disappearance (OMD)

Microbial fermentation utilizes mostly protein and carbohydrates and release SCFAs as their major products, in which carbohydrates are thought to produce more desirable products such as SCFAs than protein, which produce nitrogenous metabolites (such as phenolic compounds, amines and ammonia), some of these were found to be carcinogenic (Gibson *et al.*, 2003). Since carbohydrates are organic matter composed of carbon, hydrogen and oxygen, organic matter disappearance (OMD) might be more suitable for the evaluation of *in vitro* fermentability than determining how much dry matter, which may contains mineral residues and other inorganic matters, had been used up by the microorganisms (bacteria).

Table 3.5 showed the OMD of different β-glucans after 24h *in vitro* fermentation using human fecal innoculum. All polysaccharides or oligosaccharides tested were fermented to different extent. Generally speaking, the higher the OMD, the more the β-glucan was fermented and thus the higher was its *in vitro* fermentability. Although the OMD of all tested β-glucans were smaller than that of the positive control FOS, all of them showed higher OMD than the negative control cellulose, with significant differences noticed in the cases of PCO, LAM, CUR and CM-CUR ($p < 0.05$) (Table 3.5). As expected, LAM and PCO, which had the lowest molecular weight (about 7000 for laminarian and 360 to 1800 for PCO, details refer to Table 3.4) among the tested β-glucans, had the highest OMD. The OMD of these two β-glucans were significantly higher than that of PAC, CM-PAC, CUR and CM-CUR ($p < 0.05$).

Reducing DP in the case of PCO leaded to a significantly higher OMD than its higher DP counterpart PSS, from 22.75% ± 5.82% to 52.46% ± 6.74% ($p < 0.05$). However, CUR, with the molecular weight nearly four times higher than that of PAC (see Table 3.4), had a higher OMD, though statistically not significant ($p > 0.05$). It implied that low DP NDCs may serve as more instant sources of substrates for microbes than high DP ones. It was also found that the OMD of the CM-PAC and CM-CUR were slightly higher than their non-carboxymethylated counterparts, with an increase in OMD of 8.13% and 5.03%, respectively, though the increase was not statistically significant ($p > 0.05$). This might be due to the fact that increasing solubility of the β-glucans after carboxymethylation would enhance the accessibility of the β-glucan by the fecal microflora, which would certainly increase its *in vitro* fermentability.

Table 3.5 Organic matter disappearance (OMD) of different β-glucans and FOS upon 24h *in vitro* fermentation using human fecal inoculum at 37℃ under anaerobic condition maintained by Oxyrase

β-glucans	OMD (%)*		
PSS	22.75	±	5.82^{ag}
PCO	52.46	±	6.74^{bch}
PAC	25.21	±	5.62^{adg}
CM-PAC	33.34	±	1.15^{aeg}
CUR	37.91	±	5.67^{acf}
CM-CUR	42.94	±	9.46^{bdef}
LAM	66.59	±	5.64^{hi}
Cellulose	12.98	±	1.89^{g}
FOS	79.30	±	10.19^{i}

*Mean ± S.D. (n = 3) with different superscripts (a-i) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

OMD was seldom tested in recent prebiotic studies in which the focus have been placed on the effect of different types of oligosaccharides on fecal microflora in human volunteer studies/ cecal content in animal models, while substrate disappearance like OMD is less emphasized (Gibson *et al.*, 1995; Rycroft *et al.*, 2001; Olano-Martin *et al.*, 2002). Table 3.6 showed the OMD in one of the studies using different dietary fiber sources as the substrates (Bourquin *et al.*, 1996). It could be seen that NDPs of cereal sources like oat and wheat fiber generally had a lower OMD than those of PSS (22.75 ± 5.82%), PAC (25.21 ± 5.62%) and CUR (37.91 ± 5.67%) upon 24h *in vitro* fermentation. This might indicate that PSS and PAC (extracted from *Poria cocos* sclerotium having linear β (1→3) glycosidic linkages) as well as CUR (produced by the bacteria *Alcaligenes faecalis* var. myxogenes having linear β (1→3) glycosidic linkages) had exerted a stronger prebiotic effect than cereal dietary fiber which are glucans with β (1→3, β 1→4) mixed-linkages. However, when compared to xanthan gum and gum karaya, which contained other types of sugar residues such as galacturonic acid and rhamnose residues, PSS, PAC and CUR had a lower OMD after 24h *in vitro* fermentation. However, it should also be noted that fecal microflora was usually host-specific (Zoetendal *et al.*, 1998), the effect of various sources of NDCs on the microflora might therefore varied from one study to another.

Table 3.6 Organic matter disappearance (OMD) (%) of various NDC sources during 24h *in vitro* fermentation using human fecal microflora as illustrated in other studies

Substrate	OMD (%)*	Major linkages in the polysaccharide chain
William oat fiber	5.8	β (1→3, β-1→4) mixed-linkage glucan (Brennan & Cleary, 2005)
Canadian Harvest oat fiber	8.9	β (1→3, β-1→4) mixed-linkage glucan (Brennan & Cleary, 2005)
Corn bran	11.2	N/A**
Wheat bran fiber	25.4	β (1→3, β-1→4) mixed-linkage glucan (Jiang & Thava, 2000)
Xanthan Gum	42.3	N/A
Gum karaya	50.9	(1) repeating units of four galacturonic acid residues containing β-D-galactose branches and having an L-rhamnose residue at the reducing end of the unit (50%) (2) an oligorhamnan chain, containing D-galacturonic acid branch-residues, and interrupted occasionally by a D-galactose residue (17%). (3) D-Glucuronic acid (33%) (Raymond & Nagel, 1973)

* OMD results were adapted from Bourquin *et al.*, 1996.

** N/A: Published data not available.

3.4.2 Time course study of SCFA production

3.4.2.1 Total SCFA production

Total SCFA produced during *in vitro* fermentation using human fecal flora is usually referred to the amount of the three major SCFAs (acetate, propionate and butyrate) produced (Gibson *et al.*, 2003).

A time course SCFA production was studied and the results are shown in Tables 3.7 – 3.15. Total and individual SCFA production was calculated as the difference between the SCFA concentration (mmol /g original organic matter (OM)) of the tested β-glucans and that of the blank at a specific time interval (0h, 6h, 12h, 24h). The production of total and individual SCFAs at different time intervals could be used to illustrate the rate of *in vitro* fermentation during the incubation period. Negative values were observed in some cases, most probably at the beginning of fermentation. This situation implied that the SCFA level in the sample fermentation buffer was lower than those of the blank. At the beginning of fermentation, levels of individual as well as total SCFA were indeed comparable, and the negative values were usually small. During fermentation, the rate of the production of SCFAs during fermentation was not high enough to cover its utilization by fecal microflora in energy metabolism, resulting in temporary negative values in individual SCFA.

During the *in vitro* fermentation of the positive control FOS, SCFA concentration was the highest at 24h (5.31 ± 0.08 mmol /g original OM) (Table 3.7). However, the maximum SCFA production was observed at the first 12 hours of

fermentation of FOS (nearly 90% of the total SCFA production) rather than the last 12 hours, which only brought to an increase of less than 1 mmol/ g original OM (10% of the total SCFA production) between 12h and 24h (Table 3.7). The phenomenon might be explained by the oligosaccharide nature of FOS. FOS with low DP (DP 2 to 4) was fermented quickly in a short time, releasing most of the SCFAs in the first 12 hours of fermentation, while little was left for fermentation after 12 hours of incubation. Meanwhile, it was observed that total SCFA production at both 12h and 24h significantly differed from those of 0h and 6h ($p < 0.05$).

A different trend was observed in the cases of LAM and PCO (Tables 3.15 and 3.10 respectively), in which more SCFAs were produced during the second half of *in vitro* fermentation (from 12h to 24h) when compared to the first half (from 0h to 12h). In the case of LAM, more than 90% of the SCFAs (91.04%) were produced during the incubation period of 12h to 24h. On the other hand, about 62% of the SCFA production came from the second half of *in vitro* fermentation of PCO. LAM, with higher DP than PCO, may require longer time for the fecal microflora to ferment and produce SCFA. Therefore, the *in vitro* fermentation of PCO would release more SCFA than LAM (38% and 9% respectively) in the first 12 hours of fermentation.

In the cases of other tested polysaccharides, the fermentation of PSS, PAC, CM-PAC and CUR did not lead to significant production of SCFA ($p > 0.05$). This may be due to the fact that the rate of the production of SCFAs during fermentation was not high enough to cover its utilization by fecal microflora in energy metabolism. There was a general trend that the SCFA concentration dropped during the first six

hours of incubation (Table 3.9, Table 3.11, Table 3.12, Table 3.13 & Table 3.14). In the cases of PSS and PAC, total SCFA concentration (mmol/ g original OM) decreased from -0.29 ± 0.10 and -0.10 ± 0.04, respectively, at the beginning of incubation (0h), to -0.99 ± 0.4 and 0.83 ± 0.5 at the sixth hour (6h). The total SCFA concentration produced in CUR also dropped from -0.20 ± 0.10 at h0 to -0.63 ± 0.21 mmol/ g original OM at 6h. Initially, it might take longer time until the fecal microflora to ferment the relatively longer chain of PSS, PAC and CUR polysaccharides, followed by a gradual but insignificant increase (p >0.05) up to 12 or 24 hours of fermentation. A maximum total SCFA concentration was noticed at the twelfth hours of incubation (12h) in the case of CM-CUR (2.18 ± 1.11 mmol/ g original OM) but it declined to 1.75 ± 0.61 mmol/ g original OM at the end of the *in vitro* fermentation (24h) (Table 3.14). The trends of the production of total SCFAs at different time intervals during 24h *in vitro* fermentation of various β-glucans could also re revealed from Fig 3.10.

3.4.2.2 Individual SCFA (Acetate, Propionate and Butyrate)

Similar to the case in total SCFA production, acetate, propionate and butyrate concentrations generally decreased slightly (p > 0.05) during the first six hours of incubation in the case of PSS, PAC, CM-PAC, CUR and CM-CUR, implying that the rate of the production of SCFAs during fermentation was not high enough to cover its utilization by fecal microflora in energy metabolism. In almost all cases, significant increase (p < 0.05) in individual SCFA concentration was observed at 12h and 24h, but not at 6h. In the case of PCO and LAM, significant increase of acetate and butyratae (p < 0.05) was observed between 0h and 12h. Meanwhile, significant

difference ($p < 0.05$) was only observed in the production of acetate but not butyrate between 12h and 24h, indicating that acetate was still actively produced during the second half of fermentation, while butyrate was not. The trends of the production of acetate, propionate and butyrate at different time intervals during 24h *in vitro* fermentation of various β-glucans could also re revealed from Fig 3.7, Fig 3.8 and Fig 3.9 respectively.

Table 3.7 Individual and total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of FOS at 37℃ under anaerobic condition maintained by Oxyrase

		FOS			
SCFA	Time (h)	0	6	12	24
(mmol/g original organic matter)*					
Acetate		-0.04 ± 0.02[a]	2.12 ± 0.11[bc]	2.92 ± 0.71[b]	1.64 ± 0.37[c]
Propionate		-0.03 ± 0.01[a]	-0.13 ± 0.08[a]	0.43 ± 0.29[b]	0.76 ± 0.16[b]
Butyrate		-0.06 ± 0.02[a]	0.64 ± 0.15[a]	1.38 ± 0.08[b]	2.92 ± 0.51[c]
Total		-0.14 ± 0.05[a]	2.63 ± 0.32[a]	4.73 ± 1.04[b]	5.31 ± 0.08[c]

*Data in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.8 Individual and total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of cellulose at 37℃ under anaerobic condition maintained by Oxyrase

		Cellulose			
SCFA	Time (h)	0	6	12	24
(mmol/g original organic matter)*					
Acetate		-0.04 ± 0.02[a]	-0.16 ± 0.18[a]	0.10 ± 0.01[a]	-0.02 ± 0.18[a]
Propionate		-0.03 ± 0.01[a]	-0.30 ± 0.18[a]	-0.10 ± 0.18[a]	-0.35 ± 0.22[a]
Butyrate		-0.04 ± 0.01[a]	-0.11 ± 0.07[a]	-0.05 ± 0.09[a]	-0.17 ± 0.11[a]
Total		-0.11 ± 0.03[a]	-0.56 ± 0.39[a]	-0.04 ± 0.20[a]	-0.54 ± 0.32[a]

*Data in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.9 Individual and total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of PSS at 37℃ under anaerobic condition maintained by Oxyrase

		PSS			
SCFA	Time (h)	0	6	12	24
(mmol/g original organic matter)*					
Acetate		-0.11 ± 0.04[a]	-0.21 ± 0.2[a]	-0.15 ± 0.2[a]	-0.24 ± 0.13[a]
Propionate		-0.08 ± 0.02[a]	-0.61 ± 0.16[b]	-0.40 ± 0.2[a]	-0.49 ± 0.08[b]
Butyrate		-0.10 ± 0.04[abc]	-0.17 ± 0.06[abc]	-0.21 ± 0.07[b]	-0.04 ± 0.04[c]
Total		-0.29 ± 0.10[a]	-0.99 ± 0.4[a]	-0.76 ± 0.43[a]	-0.76 ± 0.25[a]

*Data in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.10 Individual and total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of PCO at 37℃ under anaerobic condition maintained by Oxyrase

		PCO			
SCFA	Time (h)	0	6	12	24
(mmol/g original organic matter)*					
Acetate		-0.08 ± 0.08[a]	0.93 ± 1.89[ab]	0.51 ± 0.34[a]	3.36 ± 0.79[b]
Propionate		-0.08 ± 0.04[a]	-0.85 ± 0.73[a]	-0.04 ± 0.68[a]	-0.74 ± 0.45[a]
Butyrate		-0.10 ± 0.07[a]	0.66 ± 0.28[ac]	1.03 ± 0.59[ab]	1.79 ± 0.21[b]
Total		-0.25 ± 0.19[a]	0.75 ± 2.86[ab]	1.50 ± 1.44[ab]	4.41 ± 1.24[b]

*Data in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.11 Individual and total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of PAC at 37℃ under anaerobic condition maintained by Oxyrase

		PAC			
SCFA	Time (h)	0	6	12	24
(mmol/g original organic matter)*					
Acetate		-0.03 ± 0.01[a]	-0.35 ± 0.10[b]	0.08 ± 0.09[a]	-0.04 ± 0.05[a]
Propionate		-0.03 ± 0.01[abc]	-0.41 ± 0.29[b]	0.44 ± 0.31[c]	-0.06 ± 0.30[abc]
butyrate		-0.04 ± 0.02[a]	-0.07 ± 0.12[a]	0.17 ± 0.15[ac]	0.31 ± 0.17[bc]
Total		-0.10 ± 0.04[abc]	-0.83 ± 0.50[b]	0.69 ± 0.40[c]	0.21 ± 0.51[abc]

*Data in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.12 Individual and total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of CM-PAC at 37℃ under anaerobic condition maintained by Oxyrase

		CM-PAC			
SCFA	Time (h)	0	6	12	24
(mmol/g original organic matter)*					
Acetate		-0.14 ± 0.06[abc]	-0.37 ± 0.11[b]	-0.18 ± 0.17[abc]	0.03 ± 0.20[c]
Propionate		-0.10 ± 0.03[a]	-0.44 ± 0.21[a]	-0.25 ± 0.25[a]	-0.24 ± 0.27[a]
butyrate		-0.15 ± 0.05[a]	-0.13 ± 0.07[a]	-0.12 ± 0.07[a]	-0.15 ± 0.08[a]
Total		-0.40 ± 0.14[a]	-0.94 ± 0.39[a]	-0.54 ± 0.49[a]	-0.37 ± 0.55[a]

*Data in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.13 Individual and total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of CUR at 37℃ under anaerobic condition maintained by Oxyrase

		CUR			
SCFA	Time (h)	0	6	12	24
(mmol/g original organic matter)*					
Acetate		-0.07 ± 0.04^{abc}	-0.18 ± 0.11^{b}	0.01 ± 0.10^{abc}	0.29 ± 0.26^{c}
Propionate		-0.05 ± 0.02^{a}	-0.35 ± 0.10^{a}	-0.02 ± 0.25^{a}	0.14 ± 0.34^{a}
butyrate		-0.07 ± 0.03^{a}	-0.10 ± 0.04^{a}	-0.05 ± 0.11^{a}	0.48 ± 0.16^{b}
Total		-0.20 ± 0.10^{abc}	-0.63 ± 0.21^{b}	-0.06 ± 0.43^{abc}	0.90 ± 0.73^{c}

*Data in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.14 Individual and total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of CM-CUR at 37℃ under anaerobic condition maintained by Oxyrase

		CM-CUR			
SCFA	Time (h)	0	6	12	24
(mmol/g original organic matter)*					
Acetate		0.00 ± 0.07^{a}	-0.12 ± 0.08^{a}	0.58 ± 0.38^{b}	0.71 ± 0.16^{b}
Propionate		-0.06 ± 0.07^{a}	-0.19 ± 0.20^{a}	1.15 ± 0.47^{b}	0.87 ± 0.41^{b}
butyrate		-0.08 ± 0.10^{a}	0.10 ± 0.10^{a}	0.44 ± 0.26^{b}	0.17 ± 0.05^{a}
Total		-0.14 ± 0.24^{a}	-0.21 ± 0.37^{a}	2.18 ± 1.11^{b}	1.75 ± 0.61^{b}

*Data in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.15 Individual and total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of LAM at 37℃ under anaerobic condition maintained by Oxyrase

		LAM			
SCFA	Time (h)	0	6	12	24
(mmol/g original organic matter)*					
Acetate		-0.04 ± 0.05[a]	0.15 ± 0.12[a]	2.02 ± 0.41[b]	1.25 ± 0.32[c]
Propionate		-0.03 ± 0.03[a]	0.07 ± 0.21[a]	2.54 ± 0.55[b]	1.86 ± 0.43[b]
butyrate		-0.06 ± 0.04[a]	0.26 ± 0.12[a]	2.22 ± 0.26[b]	2.56 ± 0.32[b]
Total		-0.13 ± 0.12[a]	0.49 ± 0.45[a]	6.79 ± 1.22[b]	5.68 ± 1.07[b]

*Data in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

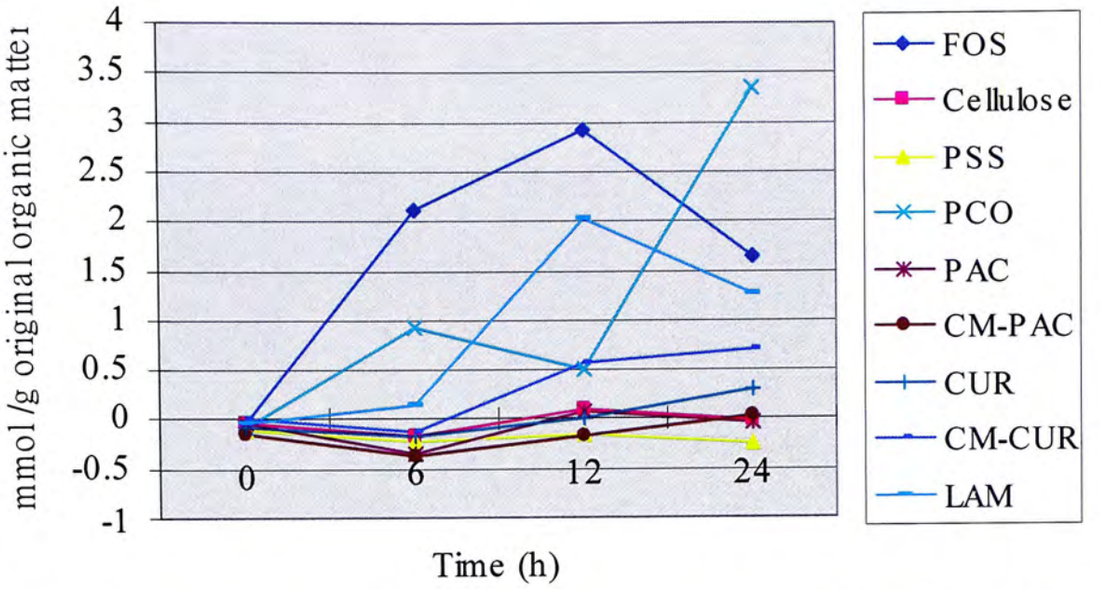

Fig 3.7 Acetate production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of β-glucans and controls at 37℃ under anaerobic condition maintained by Oxyrase

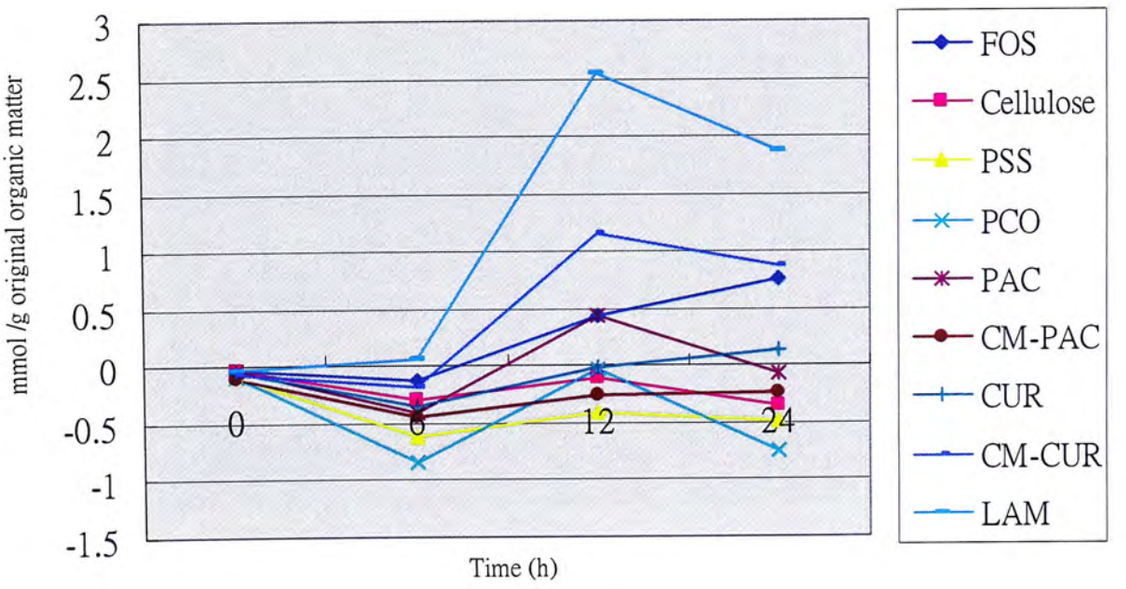

Fig 3.8 Propionate production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of β-glucans and controls at 37℃ under anaerobic condition maintained by Oxyrase

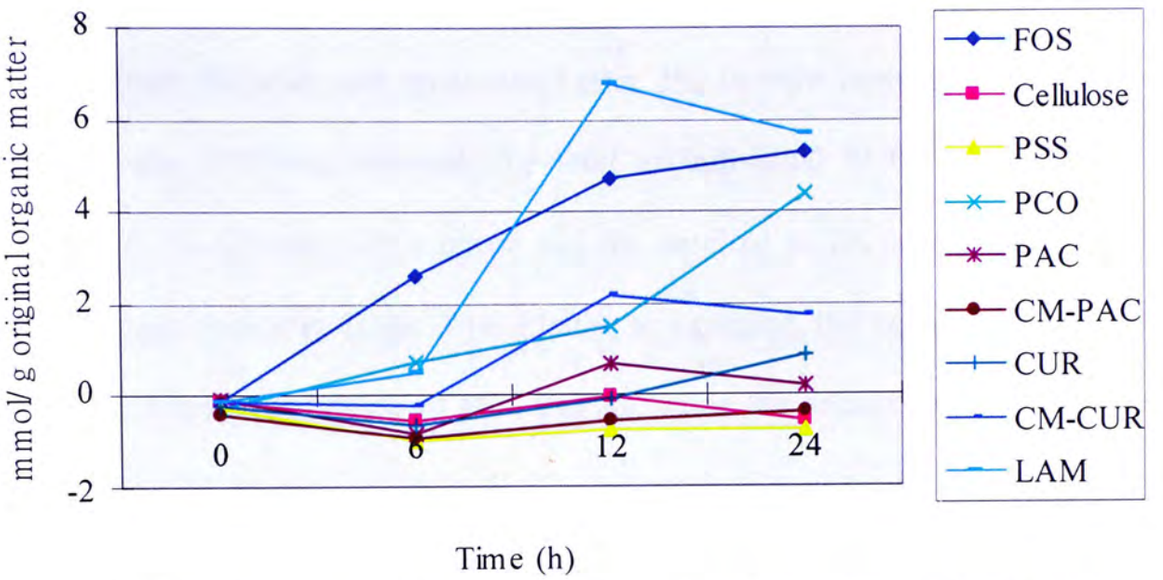

Fig 3.9 Butyrate production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of β-glucans and controls at 37℃ under anaerobic condition maintained by Oxyrase

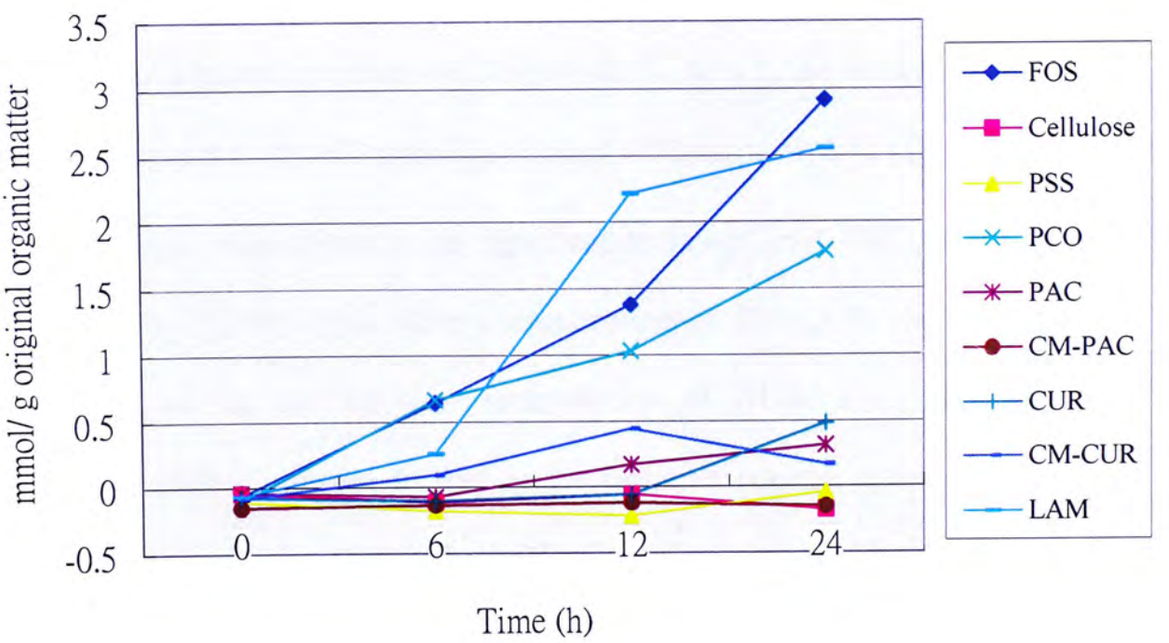

Fig 3.10 Total SCFA production at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation using 4% human fecal inoculum of β-glucans and controls at 37℃ under anaerobic condition maintained by Oxyrase

3.4.3 Overall production of total and individual SCFA

The overall SCFA production indicated the production of individual and total SCFAs (acetate, butyrate and propionate) after 24h *in vitro* fermentation. This was defined as the difference between the total or individual SCFA at 24h *in vitro* fermentation of different NDCs tested and the baseline SCFA concentration at 0h. The results are shown in Table. 3.16. Firstly, as expected, the positive control FOS showed the largest production of total SCFAs, while the negative control cellulose showed no net SCFA production at all. The net production of total SCFA after 24h *in vitro* fermentation ranged from 5.80 ± 1.09 mmol/ g original OM in LAM to -0.48 ± 0.16 mmol/ g original OM in PSS. Total SCFA production after 24h *in vitro* fermentation of PCO and LAM were significantly higher than the other β-glucans tested ($p < 0.05$) (Table 3.16). Meanwhile, total SCFA production in these two β-glucans was comparative to that of FOS ($p > 0.05$), with LAM even higher than FOS (5.80 ± 1.09 and 5.45 ± 0.12 mmol/ g original OM, respectively) (Table 3.16). Total SCFA production was found to be significantly lower ($p < 0.05$) in the cases of polysaccharides (Table 3.16). With the exception of CM-CUR, the production of total SCFAs during the *in vitro* fermentation of CUR, PAC and PSS were insignificant when compared to that of the negative control cellulose ($p > 0.05$). These may due to the fact that the high DP of these polysaccharides provided limited immediate available carbohydrates for the fecal microflora to ferment initially. The fecal microflora might then take longer time to hydrolyze these polysaccharides before SCFAs could be produced. On the other hand, a reduction of DP from PSS to PCO resulted in a four-fold increase in the total SCFA production, from -0.48 ± 0.16 mmol/ g original OM to 4.66 ± 1.36 mmol/ g original OM ($p < 0.05$).

Carboxymethylation enhanced moderately the total SCFA production in the case of curdlan (CM-CUR). Nonetheless, the effect was not observed in the case of PAC (CM-PAC). Yet, the effect was not statistically significant in both cases (Table 3.16).

Acetate was usually found to be the major SCFA produced during fecal or large intestinal fermentation (Macfarlane & Marfarlane, 2003). In the present study, however, butyrate became the major SCFA to be produced during the *in vitro* fermentation of the tested β-glucans (Table 3.16). Nonetheless, the production of acetate should not be neglected. The largest production of acetate was observed in the *in vitro* fermentation of PCO (3.44 ± 0.81 mmol/ g original OM), which was higher than that of FOS (1.68 ± 0.38 mmol/ g original OM). Acetate production during the *in vitro* fermentation of PCO was also significantly higher than PSS (-0.13 ± 0.09 mmol/ g original OM) ($p < 0.05$). It was followed by LAM (1.29 ± mmol/ g original OM), which was only slightly lower than that of FOS. Carboxymethylation enhanced the acetate production in the case of CUR and PAC. A two-fold increase of acetate production was noticed in the case of CM-CUR, and the production of this SCFA was also increased from -0.01 ± 0.04 mmol/ g original OM in PAC to 0.17 ± 0.25 mmol/ g original OM in CM-PAC (Table 3.16).

In vitro fermentation of β (1→ 3) glucans from *Poria cocos* sclerotium did not produce much propionate. All PCO, PSS, PAC and CM-PAC did not show a net increase in the production of propionate after 24h *in vitro* fermentation (Table 3.16). Propionate production in LAM was more obvious and was significantly higher than that of FOS ($p < 0.05$) (Table 3.16). A certain amount of propionate was also produced in the cases of CUR and CM-CUR, which were 0.19 ± 0.35 mmol/ g

original OM and 0.92 ± 0.46 mmol/ g original OM, respectively, though the difference was not statistically significant ($p > 0.05$).

For butyrate production, the trend was similar to that of the total and acetate production, except that the carboxymethylated polysaccharides no longer had a higher butyrate production when compared to their non-carboxymethylated counterparts (Table 3.16). Nonetheless, the differences were not significant ($p > 0.05$). On the other hand, the amount of butyrate producd in the *in vitro* fermentation of PCO was significantly larger than that of PSS ($p < 0.05$). *In vitro* fermentation of LAM produced the largest amount of butyrate (2.61 ± 0.33 mmol/ g original OM), which was comparable to that of the positive control FOS (2.98 ± 0.50 mmol/ g original OM).

Table 3.16 Overall production of total and individual SCFAs (mmol/g original organic matter) of different β-glucans and FOS after 24h *in vitro* fermentation using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

	SCFA production (mmol/ g original OM)*			
β-glucan	Acetate	Propionate	Butyrate	Total
PSS	-0.13 ± 0.09[a]	-0.41 ± 0.06[a]	0.06 ± 0.03[a]	-0.48 ± 0.16[a]
PCO	3.44 ± 0.81[b]	-0.66 ± 0.49[a]	1.89 ± 0.27[c]	4.66 ± 1.38[b]
PAC	-0.01 ± 0.04[a]	-0.03 ± 0.29[acf]	0.35 ± 0.16[a]	0.32 ± 0.49[ac]
CM-PAC	0.17 ± 0.25[a]	-0.14 ± 0.29[ad]	0.00 ± 0.12[a]	0.03 ± 0.68[ac]
CUR	0.36 ± 0.27[a]	0.19 ± 0.35[ae]	0.55 ± 0.19[a]	1.10 ± 0.78[ac]
CM-CUR	0.71 ± 0.23[a]	0.92 ± 0.46[ce]	0.25 ± 0.14[a]	1.88 ± 0.82[c]
LAM	1.29 ± 0.32[b]	1.89 ± 0.44[b]	2.61 ± 0.33[b]	5.80 ± 1.09[b]
Cellulose	0.03 ± 0.17[a]	-0.33 ± 0.22[a]	-0.13 ± 0.11[a]	-0.43 ± 0.32[a]
FOS	1.68 ± 0.38[b]	0.79 ± 0.16[def]	2.98 ± 0.50[b]	5.45 ± 0.12[b]

*Data (mean ± S.D., n=3) in columns with different superscripts (a-f) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Individual and total SCFA production upon *in vitro* fermentation of other sources of NDCs using human fecal inoculum in other similar studies are shown in Table 3.17. For substrates of high DP, i.e. oat fiber, corn bran and wheat bran fiber, acetate was the highest SCFA to be produced, followed by butyrate and lastly a very small amount of propionate was produced (Bourquin *et al.*, 1996). The same situation was observed in the fermentation of PSS, PAC and CUR. When considering the total SCFA production, the *in vitro* fermentation of CUR showed a higher amount of total SCFA produced than those of the cereal substrates, while a smaller amount of SCFA was produced in the case of PAC and PSS (details refer to Table 3.16).

In vitro fermentation of PCO and LAM seemed to produce a higher amount of SCFA (4.66 ± 1.38 and 5.80 ± 1.09 mmol/g original OM, respectively) when compared to gentio-oligosaccharides and maltodextrin (3.81 and 4.01 mmol/g original OM, respectively) (Rycroft *et al.*, 2001). However, when the positive control FOS was taken into account, it could be seen that the SCFA production between the study of gentio-oligosaccharides (Rycroft *et al.*, 2001) and the present study varied greatly. In the investigation of gentio-oligosaccharides, their SCFA production after 24h *in vitro* fermentation was actually higher than that of FOS (3.08 mmol/g original OM). But in the case of PCO and LAM, less SCFA was produced when compared with FOS (5.45 ± 0.12 mmol/g original OM). It suggested that the *in vitro* fermentability of PCO and LAM might not be as high as that of gentio-oligosaccharides.

Table 3.17 Short chain fatty acid (SCFA) production (mmol/ g original OM) upon 24h *in vitro* fermentation of various NDCs using human fecal inoculum in previous studies

Substrate	SCFA production (mmol/ g original OM)				Reference
	Acetate	Propionate	Butyrate	Total	
William oat fiber	0.28	0.09	0.12	0.48	(Bourquin *et al.*, 1996)
Canadian Harvest oat fiber	0.48	0.16	0.13	0.77	(Bourquin *et al.*, 1996)
Corn bran	0.45	0.16	0.11	0.71	(Bourquin *et al.*, 1996)
Wheat bran fiber	1.23	0.24	0.52	1.99	(Bourquin *et al.*, 1996)
FOS*	2.34	0.53	0.21	3.08	(Rycroft *et al.*, 2001)
Gentio-oligo-saccharides	3.39	0.40	0.02	3.81	(Rycroft *et al.*, 2001)

*Data about the OM of FOS, gentio-oligosaccharides was not mentioned in the study of Rycroft (2001), and was assumed to be 100% for comparision.

3.4.4 Molar ratio of SCFAs

In general, acetate, propionate and butyrate are produced in an approximate molar ratio of 60: 25: 15, but this can vary depending upon the nature of carbohydrates being fermentated (Cummings & Englyst, 1987). The molar ratio of the three major SCFAs found in humans is thus greatly influenced by dietary intake of carbohydrates.

In these project, different types of β (1→3) glucans were subjected to *in vitro*

fermentation using human fecal innoculum, resulting in different amounts of SCFA being produced. The molar ratio of the three major SCFAs after 24h *in vitro* fermentation also differed in each β-glucan and at different time intervals as shown in Table 3.18. In the following, the molar ratio of SCFA concentration would be described as acetate: propionate: butyrate. Firstly, at 0h, the molar ratio for the three major SCFAs in all cases was approximately the same, which was about 24: 15: 23 (Table 3.18). However, this ratio should not be taken as the reference value as all the individual SCFA concentrations were indeed at a very low level (Acetate: 0.17 ± 0.02 mM; Propionate: 0.10 ±0.02 mM; Butyrate: 0.15 ± 0.02 mM).

Different molar ratio of acetate, propionate and butyrate were recorded during the *in vitro* fermentation of different tested β (1→3) glucans. The ratio also differed at different time intervals, with an average ratio of 23: 33: 15 (the ratio at the beginning of *in vitro* fermentation was not taken into account), which were similar to that of the blank (28: 31: 15). There was a general trend that the concentration of propionate was the highest in the fermentation buffer, followed by acetate and butyrate. However, the production of propionate might not be solely contributed by the fermentation of the tested β (1→3) glucans, as propionate level was also high in the blank, and the net production of this SCFA by the *in vitro* fermentation of the tested β (1→3) glucans was relatively low indeed (details refer to 3.4.2 & 3.4.3). This, on the other hand, might imply that a certain amount of succinate, which was one of the metabolites formed during the break down of pyruvate, was originally present in the fecal inoculum. The succinate was then further converted to propionate by fecal bacteria during *in vitro* fermentation (details refer to Fig 1.1) (Macfarlane & Macfarlane, 2003).

It was thought that the SCFA molar ratio largely depended on the type of carbohydrate fermented. In this experiment, the average molar ratio observed in the case of β (1→3) glucans (23: 33: 15) was comparable to that of cellulose (24: 35: 15) and blank (28: 31: 15), but differed from that of FOS (27: 18: 15), in which acetate was found to be in the highest portion instead of butyrate (Table 3.18). This might be owing to the difference in their chemical structure (monosaccharide constituents as well as linkages). In the present study, FOS, with the structure Glc-α (1→2)[Fru- β (1→2)]$_n$ where n = 2 to 4, shifted the average molar ratio of SCFAs, which produced more acetate than butyrate upon *in vitro* fermentation using human fecal inoculum (Table 3.16 & Table 3.18). Meanwhile, the molar ratio of SCFAs in the fermentation medium of the tested β (1→3) glucans was not greatly altered, and was comparable to that of the blank and the negative control cellulose (Table 3.18).

Table 3.18 Molar ratio of acetate, propionate and butyrate (A: P: B) at different time intervals (0h, 6h, 12h, 24h) by *in vitro* fermentation of different β-glucans and FOS using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

Time	PSS			PCO			PAC			CM-PAC			CUR		
	A:	P:	B	A:	P:	B	A:	P:	B	A:	P:	B	A:	P:	B
0 h	25	15	23	25	15	23	26	15	23	27	15	23	25	15	23
6 h	33	37	15	26	22	15	21	35	15	21	35	15	28	39	15
12 h	26	41	15	18	28	15	23	39	15	21	39	15	23	39	15
24 h	21	31	15	26	19	15	18	28	15	28	39	15	19	27	15

Time	CM-CUR			LAM			Cellulose			FOS			Blank		
	A:	P:	B	A:	P:	B	A:	P:	B	A:	P:	B	A:	P:	B
0 h	30	15	23	24	15	22	24	15	23	25	15	22	24	15	23
6 h	22	33	15	22	31	15	25	37	15	40	21	15	34	30	15
12 h	22	40	15	22	40	15	20	29	15	29	20	15	25	26	15
24 h	29	42	15	12	19	15	28	38	15	12	12	15	24	37	15

3.4.5 Summary

The *in vitro* fermentability of various β (1→3) glucans was assessed in terms of OMD and SCFA production. The OMD and SCFA production observed were consistent to each other, in which a higher OMD would lead to a higher SCFA (total amount of acetate, propionate and butyrate) production after 24h *in vitro* fermentation. The resulted SCFA profiles, however, varied. OMD and total SCFA production of LAM was the highest after 24h *in vitro* fermentation among the β-glucans tested, and was found to be significantly higher than PAC and CUR ($p < 0.05$), which had a much higher molecular weight. Carboxymethylation of PAC and CUR enhanced the *in vitro* fermentability, as observed in CM-PAC and CM-CUR, but the effect was not statistically significant ($p > 0.05$). Reducing DP of PSS, on the other hand, led to a significant increase in the *in vitro* fermentability of this β-glucan (PCO) ($p < 0.05$).

3.5 Microbial identification and enumeration by FISH

There is currently a lack of published reports on the prebiotic or bifidogenic effect of β-glucan type NDCs, especially β (1→3) glucans. The following results focused on their bifidogenic effect and hence their prebiotic potential.

3.5.1 Time course relationship

In this experiment, the total bacterial count and the *Bifidobacteria* count in fecal samples were made by FISH. Examples of FISH images seen under fluorescent microscopes (details refer to 2.7.2.2) are shown in Fig 3.11, Fig 3.12 and Fig 3.13, representing DAPI staining of all bacteria in the fecal sample, FISH of all bacteria of the fecal sample as hybridized by Bac 338 probe as well as FISH of *Bifidobacteria* in the fecal sample as hybridized by Bif 164 probe, respectively. The fermentation mixtures with different β-glucans as substrates added were sampled at 0h, 12h and 24h and the results are shown in Tables 3.19 – 3.28.

3.5.1.1 Total bacterial count

In order to examine the reliability of FISH for bacterial quantification, DAPI was used to counter-stain the samples in addition to the Bac 338 probe. As DAPI was a nucleic acid stain, the count by DAPI was generally regarded as the total cell count and thus can be used as a validation of FISH. In general, consistent results were obtained in the counts made by these two methods.

Prior to *in vitro* fermentation (at 0h), the total bacterial count detected in the fecal inoculum by DAPI staining ranged from 2.21 ± 0.63 x 10^{10} to 6.01± 0.99 x 10^{10}cfu per g wet weight feces as compared to the by FISH that ranged from 1.95 ± 0.94 x 10^{10} to 6.92 ± 0.77 x 10^{10} cfu per g wet weight feces. The discrepancies between the counts were basically thought to be improper hybridization made, for example due to inappropriate hybridization temperature or insufficient incubation time. Differences may also be due to mistakes made during manual counting. It could be shown that counts made at the beginning of the experiment (0h) did show differences between DAPI and FISH (Tables 3.19 – 3.28), which might be due to the uneven distribution of microflora within human feces. Therefore, instead of focused on the final count, differences of the counts at different time intervals were investigated. No significant difference in total bacterial count was observed in the blank that had no substrate added (Table 3.28). The *in vitro* fermentation of FOS showed a significant increase in both DAPI as well as FISH counting ($p < 0.05$) (Table 3.19). In the case of the negative control cellulose, total bacterial count made by both DAPI and FISH was significantly reduced ($p<0.05$) (Table 3.20). However, in the fermentation of LAM, no significant increase was observed (Table 3.27) ($p > 0.05$). On the other hand, total bacterial count fluctuated significantly ($p<0.05$) during the *in vitro* fermentation of PCO (Table 3.22). The counts actually dropped between 0h and 12h ($p<0.05$) and increased dramatically in the late twelve hours ($p<0.05$). This type of fluctuation was also observed in the case of PSS, PAC and CM-PAC, though not statistically significant ($p > 0.05$). A more stable increase in total bacterial count was observed in the case of CUR and CM-CUR (Table 3.25 & Table 3.26).

3.5.1.2 *Bifidobacteria*

In recent years, NDCs (usually oligosaccharide in nature) which can induce the growth or activity of beneficial colonic microflora were regarded as prebiotics. By this definition, their ability to stimulate the proliferation of *Bifidobacteria* (bifidogenic property) is usually regarded as the most important one. In the case of the blank (Table 3.28) and the negative control cellulose (Table 3.20), *Bifidobacteria* did not increase or decrease at all. A significant increase ($p<0.05$) was observed as early as at 12h for FOS, PCO and LAM (Tables 3.19, 3.22 & 3.27, respectively). The *in vitro* fermentation of the NDCs with higher DP, i.e. PSS and PAC did not show much alteration in the number of *Bifidobacteria* in the fecal sample until 24h. Both CM-PAC (Table 3.24) and CM-CUR (Table 3.26) induce the growth of *Bifidobacteria* to a greater extent than their non-carboxymethylated counterparts (Table 3.23 for PAC & Table 3.25 for CUR), but the difference was not statistically significant ($p > 0.05$).

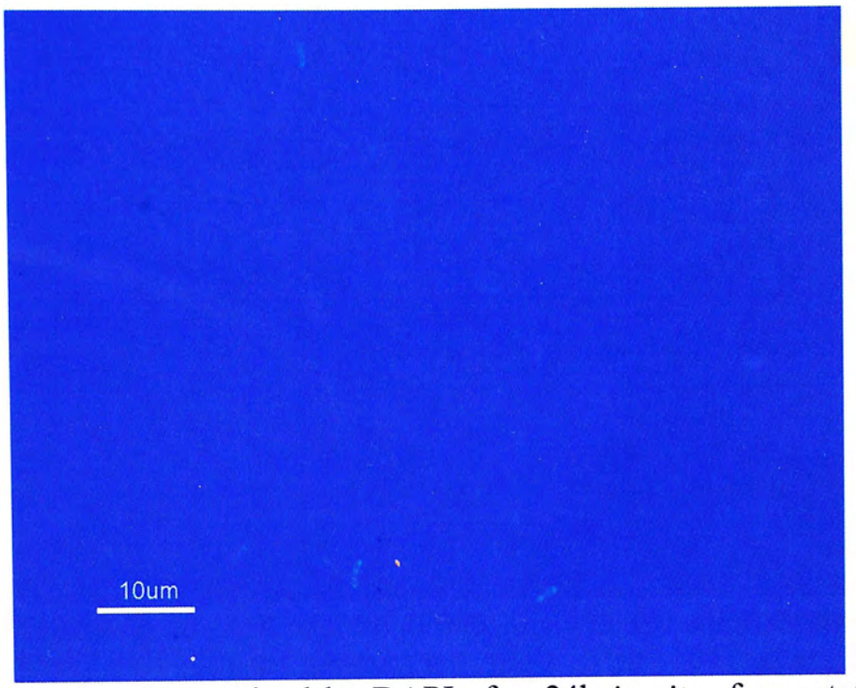

Fig 3.11 Fecal microflora stained by DAPI after 24h *in vitro* fermentation of PCO using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase (1000x)

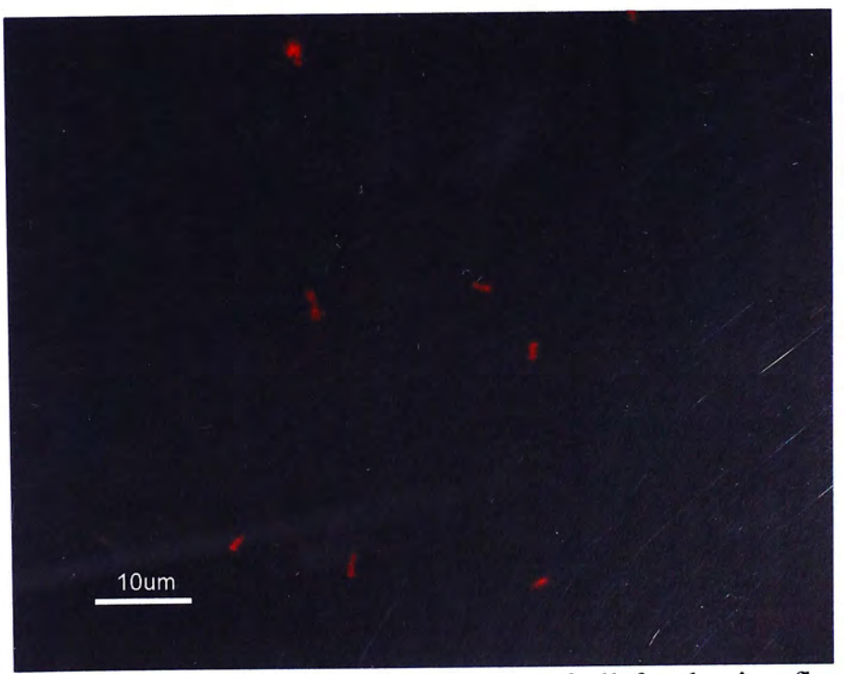

Fig 3.12 Fluorescent *in situ* hybridization (FISH) of all fecal microflora hybridized with rRNA-targeted probe (Bac 338) after 24h *in vitro* fermentation of PCO using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase (1000x)

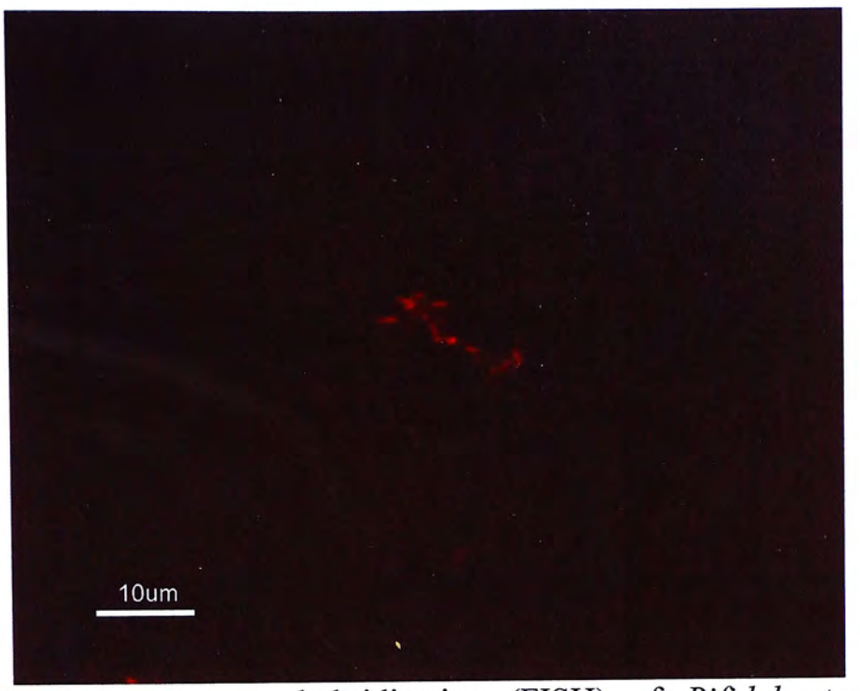

Fig 3.13 Fluorescent *in situ* hybridization (FISH) of *Bifidobacteria* in fecal microflora hybridized with rRNA-targeted probe (Bif 164) after 24h *in vitro* fermentation of PCO using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase (1000x)

Table 3.19 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of FOS using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			FOS		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		5.51 ± 1.39[a]	6.38 ± 1.01[a]	13.72 ± 0.35[b]
Total Count	Bac 338		5.77 ± 0.72[a]	6.14 ± 1.05[a]	14.25 ± 0.27[b]
Bifidobacteria	Bif 164		1.46 ± 0.21[a]	3.26 ± 0.00[b]	7.28 ± 0.15[c]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.20 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of Cellulose using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			Cellulose		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		5.52 ± 0.75[a]	4.33 ± 0.6[ab]	3.73 ± 0.50[b]
Total Count	Bac 338		5.00 ± 0.55[a]	4.44 ± 0.61[ab]	3.44 ± 0.65[b]
Bifidobacteria	Bif 164		0.80 ± 0.16[a]	0.88 ± 0.10[a]	0.79 ± 0.15[a]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations) in rows with different superscripts (a-b) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.21 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of PSS using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			PSS		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		2.21 ± 0.63[a]	3.34 ± 0.16[a]	5.79 ± 4.82[a]
Total Count	Bac 338		1.95 ± 0.94[a]	3.06 ± 0.09[a]	5.24 ± 4.07[a]
Bifidobacteria	Bif 164		1.02 ± 0.06[a]	1.04 ± 0.15[a]	1.76 ± 0.27[b]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations) in rows with different superscripts (a-b) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.22 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of PCO using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			PCO		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		4.66 ± 0.28[a]	2.78 ± 0.59[a]	7.73 ± 1.13[b]
Total Count	Bac 338		3.74 ± 0.46[a]	2.64 ± 0.52[b]	5.99 ± 0.24[c]
Bifidobacteria	Bif 164		0.73 ± 0.08[a]	1.29 ± 0.22[b]	2.68 ± 0.12[c]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations) in rows with different superscripts (a-b) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.23 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of PAC using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			PAC		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		6.01 ± 0.99[a]	2.13 ± 0.61[b]	10.96 ± 1.89[c]
Total Count	Bac 338		6.43 ± 1.49[a]	1.99 ± 0.5[b]	9.91 ± 1.29[c]
Bifidobacteria	Bif 164		0.99 ± 0.23[a]	0.75 ± 0.08[a]	1.80 ± 0.41[b]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations) in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.24 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of CM-PAC using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			CM-PAC		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		3.75 ± 0.99[a]	2.37 ± 0.48[a]	8.46 ± 0.80[b]
Total Count	Bac 338		4.07 ± 1.11[a]	2.35 ± 0.48[a]	8.97 ± 0.99[b]
Bifidobacteria	Bif 164		1.54 ± 0.31[a]	1.12 ± 0.10[a]	3.03 ± 0.51[b]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations) in rows with different superscripts (a-b) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.25 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of CUR using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			CUR		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		3.92 ± 1.76[a]	4.91 ± 0.46[a]	13.63 ± 1.30[b]
Total Count	Bac 338		4.05 ± 1.14[a]	4.56 ± 0.40[a]	9.96 ± 1.36[b]
Bifidobacteria	Bif 164		1.76 ± 0.31[a]	2.08 ± 0.37[a]	3.53 ± 0.58[b]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations) in rows with different superscripts (a-b) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.26 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of CM-CUR using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			CM-CUR		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		3.53 ± 1.21[a]	4.99 ± 0.57[a]	9.88 ± 1.03[b]
Total Count	Bac 338		2.71 ± 0.60[a]	4.44 ± 1.31[a]	9.54 ± 2.02[b]
Bifidobacteria	Bif 164		1.76 ± 0.07[a]	2.23 ± 0.29[a]	3.82 ± 0.12[b]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations) in rows with different superscripts (a-b) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.27 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of LAM using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			LAM		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		5.75 ± 1.27[a]	8.73 ± 1.75[a]	13.75 ± 1.28[a]
Total Count	Bac 338		6.92 ± 0.77[a]	8.56 ± 1.49[a]	6.92 ± 0.77[a]
Bifidobacteria	Bif 164		1.25 ± 0.16[a]	2.26 ± 0.19[b]	6.44 ± 0.32[c]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations) in rows with different superscripts (a-b) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Table 3.28 Total bacterial count and *Bifidobacteria* count* at different time intervals (0h, 12h, 24h) by *in vitro* fermentation of blank using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

			Blank		
Group	Probe/stain	Time (h)	0	12	24
Total Count	DAPI		3.12 ± 0.82[a]	3.71 ± 0.20[a]	5.66 ± 1.83[a]
Total Count	Bac 338		2.85 ± 1.08[a]	3.52 ± 0.18[a]	2.85 ± 1.08[a]
Bifidobacteria	Bif 164		1.37 ± 0.38[a]	1.80 ± 0.24[a]	1.65 ± 0.13[a]

* Data are (mean ± S.D.) x 10^{-10} cfu per g wet weight feces for 3 volunteers at 0h, 12h and 24h fermentations) in rows with different superscripts (a-c) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

3.5.2 Comparison of bifidogenic properties in the β-glucans

Finally, in order to have a comparison on the bifidogenic property of the tested β (1→3) glucans, the percentage increase in the *Bifidobacteria* number after 24h *in vitro* fermentation was assessed and compared. The results are shown in Table 3.29. As expected, the positive control FOS showed marked enhancing effect to the growth of *Bifidobacteria*, bringing a four-fold increase after 24h *in vitro* fermentation which was significantly higher than the other β (1→3) glucans (Table 3.29). A reduction in *Bifidobacteria* number was observed in the case of cellulose. On the other hand, percentage increase in *Bifidobacteria* count in cases of PCO and LAM was found to be the highest ($p < 0.05$) among the β glucans tested (Table 3.29). After 24h fermentation, percentage increase in *Bifidobacteria* was more than four times higher ($p < 0.05$) in PCO (272.56 ± 33.81 %) than that of its higher DP counterpart PSS (71.96 ± 15.23%) and PAC (81.60 ± 9.83%). PSS and PAC did not show significant difference between each other. As predicted from the SCFA production during the fermentation (details refer to 3.42 & 3.43), percentage increase in *Bifidobacteria* count was higher in the case of CUR than those of PSS and PAC, but the difference was not significant ($p > 0.05$). CM-PAC and CM-CUR did have a slight augmenting effect than their non-carboxymethylated counterparts, which was consistent with the findings in OMD and SCFA production (details refers to 3.41 and 3.42, 3.43 respectively).

Table 3.29 Increase in *Bifidobacteria* number (%)* after 24h *in vitro* fermentation of β-glucans/controls using human fecal inoculum (4%) at 37℃ under anaerobic condition maintained by Oxyrase

β-glucans/controls	Increase in *Bifidobacteria* (%)
PSS	71.96 ± 15.23^{ad}
PCO	272.56 ± 33.81^{b}
PAC	81.60 ± 9.38^{ad}
CM-PAC	97.77 ± 13.75^{d}
CUR	100.55 ± 5.18^{d}
CM-CUR	117.48 ± 5.05^{d}
LAM	309.32 ± 21.12^{b}
FOS	400.93 ± 40.47^{e}
Cellulose	-0.82 ± 1.91^{c}
Blank	23.88 ± 17.64^{ac}

*Data (mean ± S.D., n = 3) in columns with different superscripts (a-e) differ significantly (one-way ANOVA, Tukey, $p<0.05$)

Bifidogenic effect of the β(1→3) glucans tested in this project and various potential prebiotics tested in other studies relative to FOS are shown and compared in Table 3.30. Although in most of the published prebiotic studies, FOS was used as a positive control, its bifidogenic effect was found to be different in various studies, probably due to the different fermentation conditions and different fecal inoculum used in each study (Zoetendal *et al.*, 1998). It also implied that other NDCs might show different bifidogenic properties in different studies. Therefore, for comparison, the bifidogenic effect of FOS was defined to be 1. The bifidogenic effects of other NDCs investigated was calculated relative to that of FOS in the same study. The results are shown in Table 3.30. Bifidogenic effect of various high DP β (1→3) glucans, i.e. PSS, PAC, CM-PAC, CUR and CM-CUR, were obviously lower than the low DP ones i.e. melibiose, raffinose, gentio-oligosaccharides, PCO and LAM. These high DP β (1→3) glucans account for only about one-fifth to a quarter of the bifidogenic effect of FOS (Table 3.30). Bifidogenic effect of PCO was found to be lower than that of raffinose (Tzortzis *et al.*, 2004) and gentio-oligosaccharides (Rycroft *et al.*, 2001), but was comparable to that of melibiose (Tzortzis *et al.*, 2004). LAM was found to be more bifidogenic than PCO and melibiose (Tzortzis *et al.*, 2004)

PCO and LAM were found to possess bifidogenic effect towards human fecal microflora (Table 3.22 & Table 3.27), implying that human fecal microflora possess the suitable enzyme for hydrolyzing β (1→3) glycosidic bonds within PCO and LAM. Yet, the bifidogenic effect of these two β (1→3) glucans were generally found to be lower than other NDOs studied, such as melibiose and raffinose, which contained more than one type of monosaccharide constituents. It implied that hetero-

oligosaccharides might possess higher bifidogenic effect than homo-oligosaccharide ones.

3.5.3 Summary

The extent of increase in the total fecal microflora due to the treatment of the various β-glucans varied, but the bifidogenic effects of the β-glucans were found to be consistent with their *in vitro* fermentability (OMD & SCFA production). All β-glucans led to a significant increase of *Bifidobacteria* when compared to the blank and the negative control cellulose ($p < 0.05$) after 24h *in vitro* fermentation. The treatment of LAM and PCO led to an approximately three-fold increase of *Bifidobacteria* and were found to be significantly higher than other β-glucans ($p < 0.05$). Carboxymethylation enhanced the bifidogenic effect of PAC and CUR, as observed in CM-PAC and CM-CUR, but the effect was not statistically significant ($p > 0.05$). Reducing DP of PSS, on the other hand, led to a significant increase in the bifidogenic effect of this β-glucan (PCO) ($p < 0.05$).

Table 3.30 Bifidogenic effect of various potential prebiotics relative to FOS

Substrate	Composition	Bifidogenic effect relative to FOS	Reference
FOS	β-D-Fru-(1→2)-[β-D-Fru-(1→2)-]$_n$ where n=1-9	1	(Tzortzis et al., 2004); (Rycroft *et al.*, 2001)
Melibiose	6-O-α-D-galactopyranosyl-D-glucose	0.64	(Tzortzis et al., 2004)
Raffinose	O-α-galactopyranosyl(1→6)-α-D-glucopyranosyl-β-D-fructofurannoside	1.87	(Tzortzis et al., 2004)
Gentio-oligosaccharides	Glc-β(1→6)[Glc-β(1→6)]$_n$ where n = 1-5	1.06	(Rycroft *et al.*, 2001)
PSS	Glc-β(1→3)	0.18	This study
PCO	Glc-β(1→3)[Glc-β(1→3)]$_n$ where n = 2-10	0.68	This study
PAC	Glc-β(1→3)	0.20	This study
CM-PAC	--	0.24	This study
CUR	Glc-β(1→3)	0.25	This study
CM-CUR	--	0.29	This study
LAM	Glc-β(1→3) with occasional branches of Glc-β(1→6)	0.77	This study

3.6 Correlation between various parameters during *in vitro* fermentation of β-glucans

In order to test for the *in vitro* fermentability and hence the prebiotic potential of a particular carbohydrates, different parameters would be measured. As a high SCFA (acetate, propionate and butyrate) production usually indicates a higher degree of fermentation, the measurements of these three major SCFA are frequently used as indicator of fermentability. In this experiment, OMD was found to be correlated to the SCFA production ($p < 0.01$), and the highest correlation was found between OMD and the butyrate production (see Table 3.31). A high correlation (correlation coefficient: 0.894, $p < 0.01$) was also observed between OMD and the total SCFA production after 24h *in vitro* fermentation, showing that OMD could be a reliable indicator of the *in vitro* fermentability of the tested NDC. Also it provided further evidence that the SCFAs detected came from the fermentation i.e. the utilization of the added β-glucans.

For a NDC to be classified as a prebiotic, it must at least stimulate the growth of one of the beneficial colonic bacteria. In this experiment, the bifidogenic properties of beta-glucan type NDCs were investigated. The OMD of the NDCs in *in vitro* fermentation was highly correlated to the *Bifidobacteria* count (correlation coefficient: 0.916, $p < 0.01$) but not to the total bacterial count (see Table 3.32). Therefore, OMD might be an appropriate indicator for the *in vitro* fermentability as well as bifidogenic properties of the tested NDCs. It was also found that the *Bifidobacteria* number in the fecal samples was correlated to the amount of the individual as well as total SCFA produced (see Table 3.32). The correlation was found to be the highest in the case of butyrate (correlation coefficient: 0.757, $p <$

0.01), followed by acetate (correlation coefficient: 0.501, $p < 0.01$) and lastly propionate (correlation coefficient: 0.469, $p < 0.01$). It was suggested that the production of different SCFA resulted in a reduction in the medium pH, thereby suppressing the growth of most colonic bacteria. With low pH tolerance, therefore, *Bifidobacteria* could grow at a rate higher than other bacteria. (Gibson *et al.*, 2003) Moreover, the SCFA production was also found to be correlated to the total bacterial count, both made by DAPI staining and FISH (Table 3.32), suggesting that the whole colonic microflora might be altered by the SCFA composition in the colon or in the fecal sample.

Table 3.31 Correlation between DMD, OMD and short chain fatty acids production during 24h *in vitro* fermentation

	Acetate	Propionate	Butyrate	Total SCFA
OMD	**0.001*** (0.617)**	**0.001** (0.596)	**0.000** (0.912)	**0.000** (0.894)

*Figures are *p* values of correlation test (Pearson, 2-tailed) between two parameters and correlation is significant at the 0.01 level (Bolded in black).

**Figures in parenthesis represent the correlation coefficient.

Table 3.32 Correlation between bacterial count and DMD, OMD as well as SCFA production during 24h *in vitro* fermentation

	Total bacterial count by DAPI	Total bacterial count by Bac338	*Bifidobacteria*
OMD	0.883* (0.030)**	0.715 (0.075)	**0.000** (0.916)
Acetate	0.023 (0.252)	0.015 (0.270)	**0.000** (0.501)
Propionate	**0.007** (0.300)	**0.006** (0.305)	**0.000** (0.469)
Butyrate	**0.003** (0.322)	0.018 (0.263)	**0.000** (0.757)
Total SCFA	**0.002** (0.336)	**0.003** (0.322)	**0.000** (0.674)

*Figures are *p* values of correlation test (Pearson, 2-tailed) between two parameters and correlation is significant at the 0.01 level (Bolded in black).

**Figures in parenthesis represent the correlation coefficient.

Chapter 4 Conclusions

4.1 Prebiotic potential of β (1→3) glucans

Prebiotics refers to a non-digestible food ingredient that can selectively stimulate the growth or activity of one or more than one of the beneficial bacteria in human colon. *Bifidobacteria* is always regarded to be the most important bacteria that would bring beneficial effect to humans by SCFA production and immune system induction (Chaia & Oliver, 2003). Although in this project, only the changes in *Bifidobacteria* but not other bacteria during *in vitro* fermentation using human fecal inoculum were investigated, it did provide much information on the bifidogenic properties and thus insights in the prebiotic potential of the tested β (1→3) glucans.

In this project, β (1→3) glucans from different sources with different molecular weight and thus different glucose chain lengths were subjected to chemical and physical analysis and batch culture *in vitro* fermentation for the evaluation of their prebiotic potential. All PSS, PCO, PAC, CM-PAC, CUR, CM-CUR and LAM showed certain degree of *in vitro* fermentability (in terms of OMD and SCFA production) as well as bifidogenic effects when compared to the negative control cellulose, though the effects varied between the sample tested.

It was originally hypothesized that with the same amount of sample used, glucans with lower DP would always bring about a higher degree of fermentation. This hypothesis was found to be only partly correct in this project. Although the absolute molecular weight of the tested β (1→3) glucans was not known, their intrinsic viscosity [η] did provide a reliable reference that the molecular weight of

curdlan (with [η] 3.53 dL/g) could be four times higher than that of PSS or PAC (with [η] 0.89 dL/g). Nonetheless, in terms of all parameters tested, including OMD, SCFA production and bifidogenic effects, CUR obviously showed a higher potential to act as a prebiotic than PSS or PAC. This might be due to the difference in physical or chemical properties other than molecular weight and DP, such as the different conformation of polysaccharide in solution which requires further studies. On the other hand, the *in vitro* fermentability as well as the bifidogenic effect of PCO, which had a smaller DP, was higher than PSS which was the source of material of PCO. This suggested that NDCs with lower DP possess higher prebiotic potential, in terms of *in vitro* fermentability and bifidogenic effect.

Although not at a significant level, the *in vitro* fermentability (details refer to 3.4) and the bifidogenic effect of LAM was found to be higher than that of PCO. A possible reason was the slightly different glycosidic linkages found in LAM as compared to other tested β (1→3) glucans. Take for an example, cellulose has long been claimed to be poorly fermented by fecal or colonic microflora (Tungland & Meyer, 2002). The glycosidic linkages in cellulose are purely β (1→4) (Tungland & Meyer, 2002). However, the *in vitro* fermentability was found to be much higher in the case of cereal like barley, oat or wheat, which possessed mixed linkage β (1→3, 1 → 4) (Jiang & Thava, 2000). Together with its low DP, LAM with β (1→6) branched on every ten glucose molecule on the β (1→3) backbone may be more suitable than PCO to act as prebiotics.

The enhancing effect of carboxymethylation of β (1→3) glucans were observed in the *in vitro* fermentability and the bifidogenic effect, though the effect

was not statistically significant ($p > 0.05$). It was thought to be due to the higher water solubility of the carboxymethylated glucan, thereby augmenting the accessibility of the glucan to the fecal bacteria in the fermentation medium.

The present study attempted to show the prebiotic effect of various β (1→3) glucans, in which published studies about it are rare. Although a lower DP does not necessarily lead to a higher prebiotic potential, the preparation of NDOs from natural sources of NDCs does provide a way-out for novel prebiotic development in the future.

4.2 Future Work

In this project, the prebiotic potential, in terms of the *in vitro* fermentability and bifidogenic effect, was found to be significantly increased when the DP of PSS was reduced to prepare β-glucose based oligosaccharides (PCO). Hence, effect of reducing DP of CUR, which originally possessed higher prebiotic potential than PSS and PAC, is worth studying, in which the oligosaccharide prepared from enzymatic hydrolysis of CUR may possess higher prebiotic potential than PCO.

The batch *in vitro* fermentation system using human feces as the bacterial innoculum used in this project did provide useful information and insights about the *in vitro* fermentability and the prebiotic potential of the tested NDCs and hence the utilization of the tested NDCs by the human intestinal microflora. However, in order to fully mimic the real situation in the human intestine, different types of continous fermentation systems have been proposed in recent years (McBain & Macharlane, 1997). These systems try to simulate the human large intestine which possesses three

different parts of different physiological conditions, the proximal, the transverse and the distal colon. After the potential of β (1→3) glucans to be fermented in a batch system have been investigated, those with a relatively high *in vitro* fermentability and bifidogenic effect, i.e. CUR and CM-CUR, should also be subjected to *in vitro* fermentation in a continuous system so as to get a clearer picture of its action in the human large intestine.

Besides the beneficial bacteria *bifidobacteria*, other bacteria commonly colonizing the human colon such as *Bacteroides* as well as potential pathogenic bacteria like *Clostridium* and *E. coli* should also been investigated by FISH, so as to get a better understanding of the effect of these prebiotic candidates (NDCs) on the composition of the human intestinal microflora.

FISH is always regarded as a culture-independent approach that is reliable and accurate for microbial identification and quantification. However, manual counting is still labor-intensive and time-consuming. In order to simplify the hybridization procedure and shorten the time required for enumeration, multi-color FISH equipped with automated graphic processing system should be developed in near future (Takada *et al.*, 2004).

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